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The effects of BPDE and arsenite on genomic A549 DNA

by

Paula Ann Murphy

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment

of the requirements for the degree of Master of Science

in

Medical Sciences – Public Health Sciences

Edmonton, Alberta

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Abstract

Chronic ingestion of arsenic in drinking water is a cause of many illnesses such as skin, bladder and liver cancer as well as a risk factor for diabetes, hypertension, and hyperpigmentation among people in many countries. Mechanisms of action remain unclear. DNA damage can result from environmental constituents such as the carcinogen benzo[a]pyrene diol epoxide (BPDE). The body's repair mechanisms, such as the nucleotide excision repair mechanism must repair this damage to prevent the accumulation of errors. An objective of this research was to study BPDE-DNA damage and the effect of arsenic on the induction and repair of this damage. To accomplish this, the first component of this research was to develop a sensitive assay for measuring BPDE-DNA damage. A fluorescent affinity approach was investigated using capillary electrophoresis laser-induced fluorescence. Parameters such as capillary length, separation buffer concentration, DNA denaturation conditions and electrophoresis field strength were investigated. Both the competitive and non-competitive assays were sufficient for determining BPDE-DNA adducts in A549 human lung carcinoma cells. The effect of incubating A549 cells with BPDE alone or BPDE in conjunction with arsenite, were investigated. The repair of BPDE-DNA damage for a period of 0–24 hours was compared. Preliminary results showed that treatment of A549 cells with BPDE and arsenite in combination appeared to result in more damage and/or less repair than treatment of cells with BPDE alone.

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List of Abbreviations

As	arsenic
B(a)P	benzo(a)pyrene
BPDE	benzo(a)pyrene-7,8-diol-9,10-epoxide
CE	capillary electrophoresis
DMA^{V}	dimethyl arsonic acid
DMA^{III}	dimethyl arsonous acid
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
fbs	fetal bovine serum
g	gram
GC	gas chromatography
HPLC	high performance liquid chromatography
i.d.	internal diameter
IgG	immunoglobulin G
L	liter
LIF	laser-induced fluorescence
μ	micro
m	milli
M	molar
MMA^{V}	monomethylarsonic acid
MMA^{III}	monomethylarsonous acid
MS	mass spectrometry
NaOH	sodium hydroxide
NER	nucleotide excision repair
OR	odds ratio
PAH	polyaromatic hydrocarbon
PBS	phosphate buffered saline
ppb	parts per billion (μg/L)
ppm	parts per million (mg/L)

SMR	standardized mortality ratio
TMR	tetramethylrhodamine
UV	ultraviolet
V	volts
XP	xeroderma pigmentosum

Chapter 1. Introduction

Carcinogenesis is a complex multi-step process that can progress toward clinical manifestations of cancer such as neoplastic cell growth and tumorigenesis. Exposure to carcinogenic chemicals in the environment can result in cancer if repair mechanisms fail. Some carcinogenic chemicals are capable of binding covalently to DNA when metabolically activated. If the resulting carcinogen-DNA adduct is not repaired before replication a mutation may occur. The mutation may then be retained in future replication. The binding of a carcinogen to DNA is thought to be one of the essential steps in early chemical carcinogenesis. (Friedberg, 1995). The development and potential progression of cancer can be influenced by the frequency of exposure, concentration and amount of carcinogen to which an individual is exposed as well as the genetic predisposition of the individual.

1.1 BPDE

Benzo(a)pyrene (B(a)P) is a polyaromatic hydrocarbon that is commonly found in the environment. Benzo(a)pyrene, is found in coal smoke, and cigarette smoke and as a by-product of the combustion of fossil fuels (ATSDR, 1990). Early animal studies involving the application of coal tar to the backs of rabbits and mice resulted in tumours (Fang et al., 2001). Benzo(a)pyrene was identified

as a major carcinogen in coal tar in 1930; further studies allowed the identification of benzo(a)pyrene-DNA adducts in internal organs and showed that different modes of

exposure resulted in differences in the quantities and types of resulting adduct (Fang et al., 2001).

1.1.1 Nucleotide excision repair

BPDE, (±)-anti-r-7,t-8-dihydroxy-t-9,10-epoxy-7,8,9,10,tetrahydrobenzo(a)-pyrene, is a carcinogenic metabolite of benzo(a)pyrene and the species considered to be the ultimate carcinogen (Figure 1), (Fang et al., 2001, Szeliga and Dipple, 1998). *In vivo* metabolism of benzo(a)pyrene occurs predominantly in the liver by epoxide hydrolase and cytochrome P450 to produce BPDE. Bulky BPDE-DNA adducts are then formed from the covalent reaction of BPDE with the N^2 position of deoxyguanosine. The resulting bulky adducts are a form of DNA damage that if not repaired, can result in altered nucleotide sequences during replication; these alterations may initiate carcinogenesis by activating or inactivating genetic targets such as oncogenes and tumour suppressor genes (Venkatachalam et al., 1995, Denissenko et al., 1996).

Repair of BPDE-DNA adducts occurs through the NER (nucleotide excision repair) pathway, (Figure 2). NER recognizes a wide range DNA damage caused by various sources such as UV, X-rays, and benzo(a)pyrene (Klug and Cummings, 1996, Sancar, 1994, Hoeijmakers, 2001, Wani et al., 2000). Nucleotide excision repair is regarded as the major pathway for removal of a wide variety of bulky DNA adducts (Hartwig et al., 1997). Figure 2 shows a schematic diagram depicting various components of the NER pathway. A general description of NER follows (Klug and Cummings, 1996).

1. Incision/Excision step: The distorted strand containing the bulky DNA adduct is recognized and excised by a nuclease that results in a gap in the DNA helix.
2. Filling the gap: DNA polymerase I fills the gap left by excision with nucleotides complementary to those on the intact strand.
3. Sealing the gap: DNA ligase seals the gaps where nucleotides have been inserted into the strand.

Though genetic damage may occur, repair processes ensure that carcinogenesis does not always occur. Failure to repair genetic damage and the production of a carcinogenic metabolite can contribute to the carcinogenic process. The level of DNA adducts is also influenced by the sum of adduct formation and adduct removal. The concurrent activities of metabolic activation of a carcinogen and enzymatic removal of the carcinogen-DNA adduct can determine the rate of adduct removal (Li et al., 2001) as well as the development of carcinogenesis.

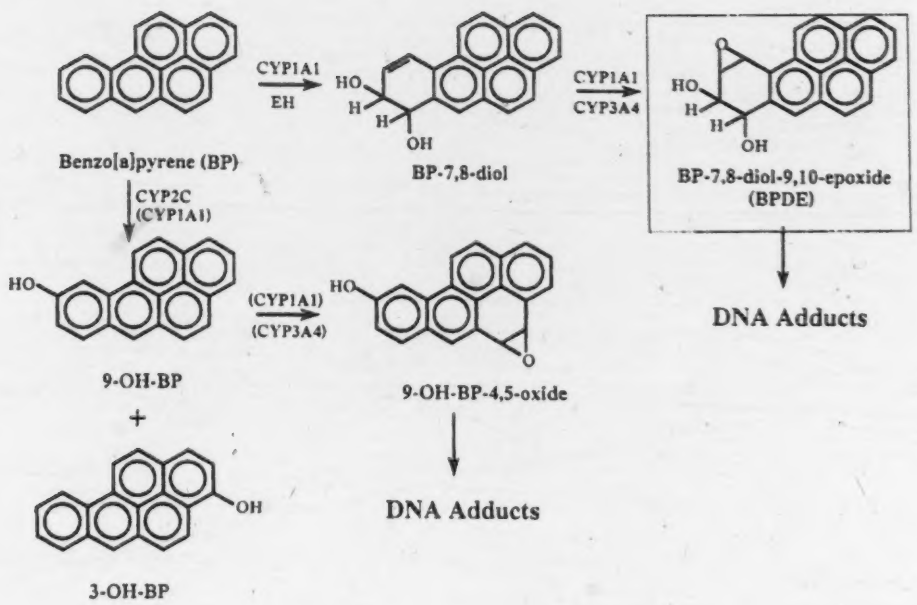

Figure 1. Benzo(a)pyrene-7,8-diol-9,10-epoxide, the ultimate carcinogen of benzo(a)pyrene, is shown in the box.

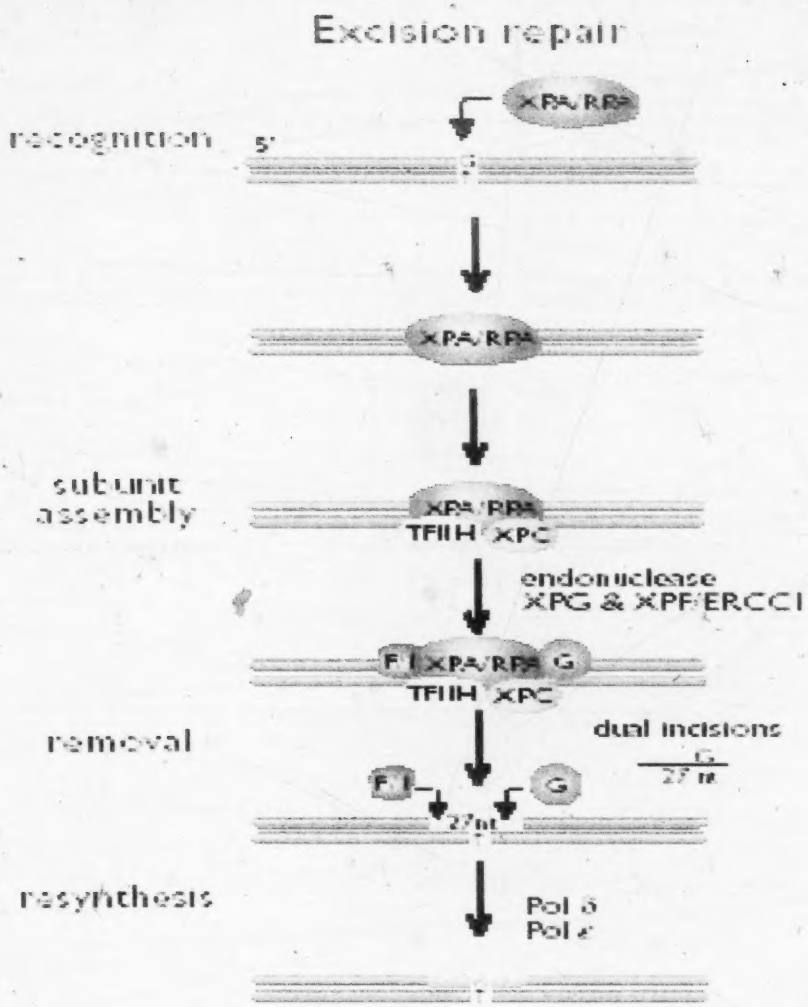

Figure 2: Diagram of nucleotide excision repair (Sancar, 1999).

1.2 Factors influencing the development of cancer

Other factors that influence whether or not cancer will occur are sex, ethnicity, genetic predisposition, and occupational exposure. Goldman et al., (2001), for example, found that lung cancer rates were higher among African-American men followed by Caucasian males; both African-American females as well as Caucasian females have lower lung cancer rates than men. In addition to this, PAH (polycyclic aromatic hydrocarbons) concentrations were higher in the lung tissues of men than women even though smoking behaviors were similar for both groups (Goldman et al, 2001). Cigarette smoke is an important source of benzo(a)pyrene. Though benzo(a)pyrene is metabolized to a carcinogenic form and the smoker is exposed to this carcinogen, only 10-15% of heavy smokers develop lung cancer. This suggests variable genetic susceptibility to lung cancer among those who smoke (Li et al., 2001). Interindividual variability may also greatly influence response to BPDE exposure. Li et al., (2001), found a four-fold difference in relative BPDE-DNA adduct levels among lymphocytes isolated from four individuals and treated with 4uM BPDE for 4 hours. The lowest relative adduct level was approximately 20 adducts per 10^7 nucleotides in one individual while lymphocytes from another individual showed the highest level at approximately 80 adducts per 10^7 nucleotides (Li et al., 2001). This sample size not large enough to draw any statistical conclusions but demonstrates that variability may exist in adduct levels among individuals. Another study has shown inter-individual variations of PAH-DNA adducts ranging from 10 to 100-fold were demonstrated after exposure to similar levels of PAHs (Rojas et al., 2000).

Occupational exposures, such as coke-oven work, pesticide manufacturing; sodium arsenate for example, is used in ant killers and in animal dips as an insecticide, and is used in smelting operations and aluminum work for the production of semiconductor materials. (Goldman et al., 2001; NIEHS, Report on Carcinogens, 1999).

1.2 Arsenic

Arsenic is naturally present in the environment. Both inorganic arsenate and arsenite, an inorganic form of arsenic can be found in drinking water (NRC, 1999 and 2001). While arsenic exists in many forms the trivalent and pentavalent states are of most concern in relation to human health (Hughes, 2002). The inorganic forms of arsenic are classified by the IARC (International Agency For Research on Cancer) as known carcinogens (IARC, 1980). In parts of Taiwan, China, South America, Bangladesh, and the West Bengal region of India, arsenic is found in drinking water at concentrations that can exceed 2000μg/L (NRC, 2001). The former maximum contaminant level (MCL) in the United States was 50 ppb but the USEPA (United States Environmental Protection Agency) has reduced the MCL to 10 ppb (EPA, Arsenic Rule 2001).

1.2.1 Metabolism of arsenic

Common routes of exposure to inorganic arsenic are oral and inhalation. Oral ingestion of arsenic in drinking water is the most common source of non-occupational

exposure and millions of people worldwide are at risk from such exposure (Liu et al., 2001; NRC, 1999; CDC, 2001; World Health Organization, 2001).

Occupational exposures are mostly inhalation exposures and occur through smelting, semiconductor and glass manufacturing, as well as power generation from the burning of arsenic-contaminated coal (Liu et al., 2001). Chronic exposure to inorganic arsenic can also result in skin lesions, diabetes, hypertension, Blackfoot disease, liver injury peripheral neuropathy, and bone marrow depression among other illnesses (Hughes, 2002). Epidemiological evidence has correlated chronic exposure to inorganic arsenic with increased incidence of skin, bladder, and lung cancer (Liu et al., 2001; NRC, 2001)

The metabolism of arsenic varies widely among species. The metabolic pathway of most mammals results in the formation of MMA (monomethyl arsenic) and DMA (dimethyl arsenic) after a series of methylation steps. Some mammals, such as chimpanzee, marmoset, and guinea pigs, do not efficiently methylate arsenic as they lack enough of or are deficient in the enzyme methyltransferase, which is responsible for methylation. Many details of the arsenic methylation process are still unknown. Figure 3 shows a general schematic of the process.

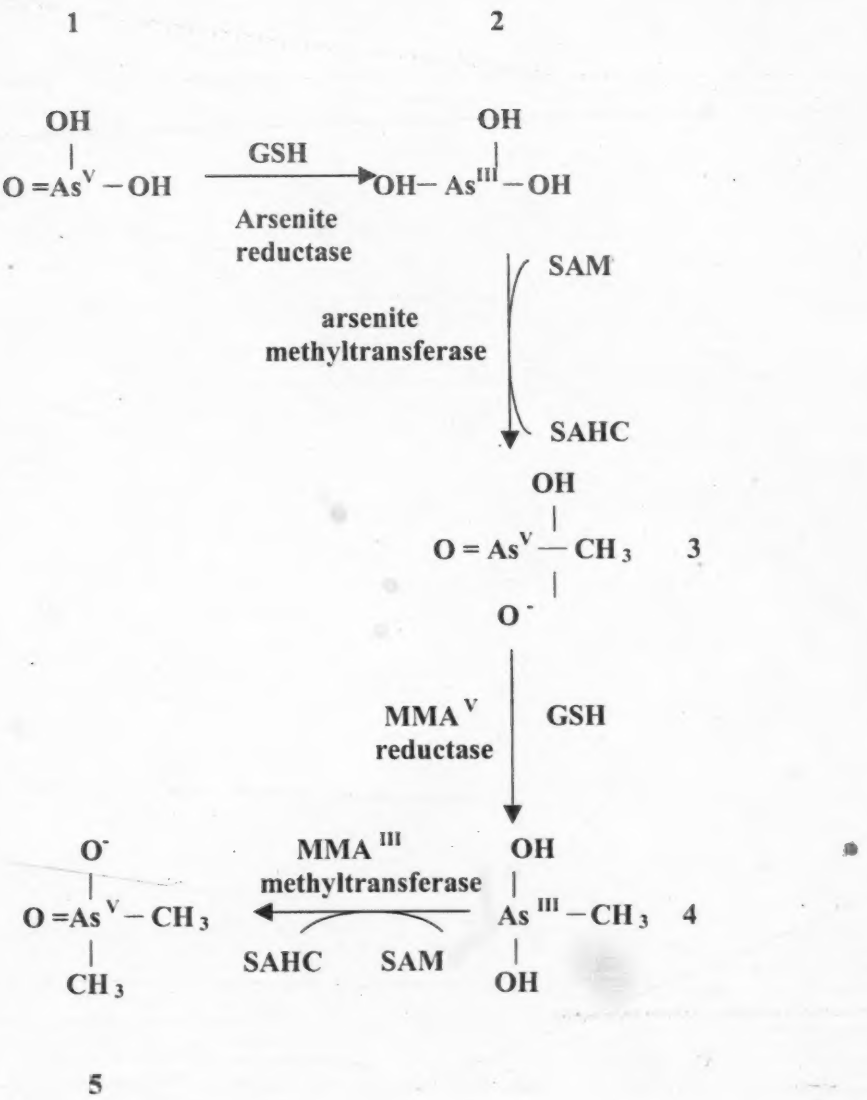

Figure 3. Biotransformation pathway for inorganic arsenic. SAM is S-adenosylmethionine and SAHC is S-adenosylhomocycteine. Numerical labels correspond to the following: 1. arsenate; 2. arsenite; 3. monomethylarsonic acid; 4. monomethylarsonous acid; 5. dimethylarsinic acid.

Previously, the accepted model of arsenic viewed MMA^{III} as a less toxic metabolite of than inorganic forms of arsenic and methylation was seen as part of the detoxification process. In light of new results by Le et al., (2000), Aposhian et al., (2000) and Petrick et al., (2001) for example, aspects of arsenic's biotransformation model have been changed. Experimental results have detected MMA^{III} in urine of chronically exposed persons (Aposhian et al., 2000) and also in rat liver bile (Gregus et al., 2000). It is now clear that MMA^{III}, once thought to be an intermediate of the biotransformation process is a stable metabolite. Aposhian et al., (2000), found MMA^{III} in urine of individuals chronically exposed to inorganic arsenic in Inner Mongolia who had been given DMPS, a chelation agent. MMA^{III} was detected only after DMPS therapy; it was not known whether MMA^{III} was a result of reduction of MMA^{V} or endogenously produced (Aposhian et al., 2000) Increased sensitivity of a HPLC-hydride generation/ atomic fluorescence spectrometry method in our lab allowed improved detection and identification of MMA^{III} as endogenously produced (Le et al. 2000, Le et al. 1998). Petrick et al., (2001), injected 66 male golden Syrian hamsters with MMA^{III} and 78 of the same with sodium arsenite intraperitoneally. The animals were observed for mortality during the 24 hours following injection. The LD_{50} (lethal dose / concentration of a test compound at which 50% of the test animals do not survive) of MMA^{III} was 29.3µg/kg body weight while that of arsenite was 112 µg/kg body weight. Death for hamsters receiving MMA^{III} occurred within 2 to 12 hours after MMA^{III} administration; arsenite mortalities occurred later at between 12 and 24 hours (Petrick et al., 2001). The previous view of methylation as a detoxification pathway needs to be rethought as a result. Previously, inorganic arsenicals (III and V) were viewed as being more toxic than the

organic forms. Current results have shown that this is not the case. It appears that the valence state may contribute to toxicity with trivalent arsenic compounds being more toxic than pentavalent compounds (Petrick et al., 2001).

Many questions still remain about the roles and effects of arsenic species in the human body. Aposhian et al., (2000), analyzed urine samples from 58 subjects in Romania who were exposed to four levels of arsenite in their drinking water (2.8, 28.5, 85.5, 161.2 μg arsenic/ L). MMA ^{III} was detected in the urine of only 17% of participants in addition to this the amount of urinary MMA^{III} was dose-dependent on the amount of arsenic in drinking water. The authors (Aposhian et al., 2000) suggest that some populations may process arsenic differently and may therefore be more susceptible to the effects of arsenic. Another possibility introduced by the new insight into arsenic's metabolism is whether or not the urinary concentration of arsenic depends on the intracellular concentration of MMA^{V} (Aposhian et al., 2002). MMA^{V} reductase is required for the production of MMA^{III}; this rate-limiting enzyme will convert MMA^{V} to MMA ^{III} when the concentration of MMA^{V} is in the millimolar range. At lower concentrations MMA^{V} will be expected to be bound to tissues or excreted unchanged, with increasing arsenic exposure the toxic metabolite MMA^{III} is produced and may then produce toxic effects (Aposhian et al., 2002). These new contributions to the understanding of arsenic make it clear that much more work is needed to fully understand the role of arsenic in human health.

1.2.2 *In vivo* and *in vitro* experiments

While the metabolism of arsenic has been well established the carcinogenic mechanism of action *in vivo* has not been determined. The inability to obtain consistent results with animal models has somewhat hindered the investigation into arsenic's effects *in vivo*. *In vitro* studies have shown that various rodent cells lines, including Chinese hamster V79 cells, appear to have an inducible tolerance to arsenic exposure that protects against development of tumors from arsenic exposure (Gebel, et al., 2001). *In vitro* studies have shown many human cell lines to be more sensitive to arsenic exposure than rodent cells such as Chinese hamster cells and that human keratinocytes are especially more sensitive to damage from arsenic exposure (Rossman et al., 1997). Rodents, commonly used for *in vivo* studies, do not readily exhibit tumor formation as a result of exposure to arsenic and while it is difficult to extrapolate *in vitro* to *in vivo* studies, the above mentioned *in vitro* results may provide a clue to the difference between human and rodent tumor development in this case (Rossman et al., 1997).

In vivo studies using rodents have shown many negative and still fewer positive results. Mice exposed to 10 parts per million arsenite for 26 weeks in their drinking water did not develop tumors (Rossman et al., 2001). Studies in rats, dogs, and monkeys have shown no carcinogenic effect after a lifetime of exposure to 5 ppm arsenite in drinking water and a diet including 5-125 ppm arsenite and 5-250 ppm arsenate (Kanisawa et al. 1969; Byron et al., 1967; Thorgiersson et al., 1994). Several studies in the 1980s showed that exposure to arsenic trioxide could result in carcinogenicity. Hamsters were exposed

to doses with 3.75 or 5.25 mg of arsenic once per week for 15 weeks and monitored for signs of carcinogenesis throughout their lifespan (Pershagen et al., 1984; Yamamoto et al., 1987); mice treated with As_2O_3 showed up to 50% mortality and adenocarcinomas, carcinomas, and adenomas in the respiratory tract.

1.3 Arsenic health effects

In view of the lack of available direct *in vivo* studies, analytical methods such as the one employed in this thesis (CE-LIF mentioned above) and epidemiological studies are the best available means to understand the effects of arsenic on humans. Epidemiological studies provide insight into many variables for example, concentration effects, and clinical manifestations that in turn may allow an understanding of arsenic's mechanism(s) of action on populations (see Tables 1 and 2 for some well known epidemiological studies). For example, Tsuda et al., (1995) conducted a historical cohort for 33 years in the area of Niigata Prefecture in Japan that resulted in many useful insights into the effects of ingesting drinking water contaminated with arsenic on human health. Of the 454 participants, those who ingested water containing an arsenic concentration greater than 1ppm demonstrated a greater number of internal cancers than statistically predicted as normal (Tsuda et al., 1995). The exposure an individual receives is not always easily measured and skin signs such as hyperkeratosis, hyperpigmentation and white spots can be used as signs of exposure and experimental results indicate that clinical signs are associated with a high risk for cancer (Tsuda et al., 1995). One further useful piece of information obtained from this epidemiological study was evidence of a

synergistic relationship between arsenic and smoking in the development of lung cancer (Tsuda et al., 1995).

Table 1. Noncancerous health effect of arsenic. Table shows source, exposure, and health effects for some epidemiological studies conducted on populations exposed to differing arsenic levels.

System or Organ Affected	Source of exposure and concentrations in exposure groups	Health effect	Authors, Country of study
Cardiovascular	Drinking water (<500μg/L, 500-1000μg/L,1000μg/L)	Hypertension in all groups	Rahman et al., 1999. Bangladesh
Dermatological	Drinking water Males (<150μg/L, >1000μg/L) Females ((<150μg/L, >1000μg/L) Drinking water Mean concentration values: 14.0± 3.1μg/L 116.0± 37μg/L 239.0± 88μg/L	Drinking water Age adjusted pre-valence of skin lesions* (18.6/100, 37.0/100) (17.9/100, 24.9/100) 26% of those in middle and high exposure groups (116.0±37 – 239.0±88μg/L) showed at least one lesion	Tondel et al., 1999, Bangladesh Hernandez-Zavala, 1999, Mexico
Hematological	Drinking water Range of concentrations 700 –930μg/L	Incidence of diabetes mellitus for those 55 years and over was significantly higher than for control groups	Tseng et al., 2000 Taiwan

*hyperpigmentation, melanosis and blackfoot disease

Table 1 continued.

System/Organ affected	Source of exposure and concentrations in exposure groups	Health effect	Author, country of study
Respiratory	Drinking water (136-1000μg/L) males exposed: 14/40 not exposed: 11/50 females exposed: 15/54 not exposed:2/74	Chronic bronchitis	Milton et al., 2001, Bangladesh
Liver	Drinking water Mean concentration values: 14.0± 3.1μg/L 116.0± 37μg/L 239.0± 88μg/L	Group exposed to 239.0± 88μg/L showed increased serum alkaline phosphatase and bilirubin Inconclusive health effect	Hernandez-Zavala, 1999, Mexico
	69 patients diagnosed with chronic arsenic poisoning	Portal fibrosis 91.3% Cirrhosis 2.9% Normal histology 5.8%	Santra et al., 1999 India

Table 2. Some types of cancer associated with arsenic ingestion and associated statistics from source epidemiological studies. (Table adapted from Arsenic in Drinking Water: 2001 Update, National Academy Press).

Cancer type	Exposure route and concentration	Outcomes	Authors, Country site
Lung	Public water supply 0 - 10μg/L 10-29μg/L 30-49μg/L 50-199μg/L 200-400μg/L Blackfoot endemic area (water supply)	Odds ratios[a] 1 1.6 (0.5-5.3) 3.9 (1.2-12.3) 5.2 (2.3-11.7) 8.9 (4.0-19.6) Male mortality: 332, SMR[b]:3.2 Female mortality: 233, SMR: 4.13	Ferreccio et al., 2000, Chile Chen et al., 1985, Taiwan
Bladder	Drinking water <0.1μg/L(low) 0.1–0.5μg/L (medium) >0.5μg/L(high)	61 cases in total 23 cases (low) 19 cases (medium) (OR=53)** 19 cases (high) (OR=2.44)**	Kurtiso et al., 1999 Finland
Urinary tract cancer	Household well water <10μg/L 10 - 50μg/L 50.1 – 100μg/L >100μg/L	Incidence rate/100000 (OR)** 37.6 4.8 (1.5) 66.4 (2.2) 134 (4.8)	Chiou et al., 2001, Taiwan

Table 2 continued.

Kidney	Blackfoot endemic disease area	Male mortality 42 SMR: 7.72 Female mortality 62 SMR: 11.19	Chen et al., 1985, Taiwan
	Drinking water	SMR for exposure groups (M:male, F:female) Low:M 0.87;F 1.24 Medium: M 1.33; F 1.34 High: M 1.57; F 2.16	Hophayn-Rich et al., 1996 – 1998, Argentina

**OR =Odds ratio, study uses a reference group of 275 age and sex matched people from the same population; [a] referent population exposed to 0-10μg/L; [b] SMR: Standardized Mortality Ratio

Though arsenic is well recognized as a poison, it is also used as a treatment for acute promyelocytic leukemia (APL) in the form of arsenic trioxide (As_2O_3). The typical treatment for APL involves all-trans-retinoic acid therapy (ATRA) with or without chemotherapy to induce remission; this therapy results in complete remission in 70% to 95% of patients but relapse occurs in about 20% to 30% of patients who are mostly resistant to further ATRA treatment (Kwong et al., 2001). Three separate studies have shown that As_2O_3 resulted in complete remission in 29 of 34 patients who had relapsed (Kwong et al., 2001). One multicenter clinical study involved 40 APL patients who had relapsed or were resistant to ATRA therapy; patients were given Trisenox (As_2O_3 trademark name), in 0.15mg/kg/day doses intravenously over 1-2 hours daily for up to 60 days until no leukemia cells were apparent in bone marrow (Coren et al., 2001). Twenty eight of forty patients showed complete remission with a median time to remission of 50 days (Coren et al., 2001). Effects from arsenic trioxide treatment resulted in mild and reversible toxicity and currently, long term health effects from As_2O_3 treatment are unknown (Kwong et al., 2001).

1.3.1 Mechanisms of action

Various methods have been proposed to help explain arsenic's effects on DNA. Hartwig et. al (1997) found that arsenite altered DNA repair. Concentrations as low as 2.5 μM arsenite impaired DNA repair in Hartwig's work (1997). Using normal cells (VH16 and VH25) and the repair-deficient XPC (xeroderma pigmentosum) fibroblasts treated with UV radiation and then arsenite, Hartwig et al. (1997) found that the NER incision frequency decreased by 30-40% and that ligation was most often affected at

cytotoxic doses of arsenite (20-50 μM) (Hartwig et al., 1997). Excision and ligation are crucial steps in nucleotide excision repair.

Another of these proposed mechanisms of action is that inorganic arsenic is a co-mutagen with some chemicals and UV light in mammalian cells. DNA excision repair of thymine dimers in human fibroblasts is inhibited by inorganic arsenic, with sodium arsenite being the most potent (Okui et al., 1986). Exposure to sodium arsenite has been shown to result in the induction of strand breaks and aneuploidy (Basu et al., 2001).

Other studies suggest that DNA methylation is altered by arsenic and that such alterations may have a role in the development of cancer. Mass et al., (1997) using A549 cells, found that arsenite increased the resistance of the p53 gene promoter region to cleavage by a restriction enzyme, HpaII. This means that an increase of methylated cytosine was present in the p53 gene. This in turn may result in modified gene expression (Mass et al., 1997). Modified gene expression in the p53 gene can result in the loss of tumor suppression.

Co-carcinogenesis is offered as another explanation for the effects of arsenic. Survival rates in animals dosed with arsenic trioxide alone, benzo(a)pyrene alone, or both arsenic trioxide and benzo(a)pyrene were 73%, 84% , 62% respectively and 78% in the control group (Pershagen et al., 1994). When animals were exposed to both arsenic trioxide and benzo(a)pyrene respiratory tract carcinomas were found in 25 out of 54 animals (Pershagen et al., 1984). In contrast, 14 out of 40 of those animals exposed to

benzo(a)pyrene and 3 out of 47 control animals show these carcinomas (Pershagen et al., 1994). In addition to this, animals that had received both carcinogens simultaneously showed more pulmonary lesions than animals treated with each chemical alone (Pershagen et al., 1994). Rossman et al. (2001) showed that arsenic is a co-carcinogen with UV radiation on mouse skin. Mice exposed to 10,000 mg/L arsenic in their drinking water and UV radiation had a 2.4-fold increase in tumors than mice exposed to UV radiation only (Rossman et al., 2001).

Chronic exposure of residents in Inner Mongolia, China to tube-well water with high concentrations of arsenite has been shown to induce oxidative stress (Pi et al., 2002). Using a high and low exposure group who had ingested water with 410μg/L and 20μg/L respectively for 18 years, Pi et al., (2002) found that the group exposed to high arsenic levels also showed a higher level of lipid peroxidase (LPO), an enzyme involved in the production of reactive oxygen species. These increased levels of LPO may result from the overproduction of reactive oxygen species (ROS) in response to high levels of arsenic from chronic exposure (Pi et al., 2002). Experimental results by these researchers have found that LPO concentrations were significantly correlated with blood levels of arsenic and its metabolites (Pi et al., 2002). Reactive oxygen species are detected in human-hamster hybrid (A_L) cells within 5 minutes after exposure to arsenite (Pott et al., 2001). Chromosomal deletions and sister chromatid exchanges result in fibroblasts exposed to arsenite (Hughes, 2002) but A_L cell experiments with 0.1% DMSO, a radical scavenger, and 2000μg/ml of arsenite showed one-third the level of reactive oxygen species in comparison to A_L cells exposed only to 2000 μg/ml arsenite (Liu et al., 2001).

These are a few of the many hypotheses offered as potential mechanisms for arsenic's action in animals and *in vitro*. While it is possible that arsenic may act to a certain degree in all of these ways; further experimentation may allow a better understanding of why arsenic can be both harmful and beneficial.

1.3 BPDE and Arsenite

In 1999, the Agency for Toxic Substances and Disease Registry (ATSDR) and the Environmental Protection Agency compiled a list of hazardous constituents in the environment. Based on frequency of occurrence, toxicity, and potential exposure, arsenic was listed as the top metal and benzo(a)pyrene topped the list for PAHs (ATSDR, 1999; EPA, 1999). PAHs such as benzo(a)pyrene can increase biotransformation into active forms with the help of P450s; the role of metals in this biotransformation may provide insight into the effects of metal/PAH mixtures commonly found in the environment. Vakharia et al., (2001) found that metals, such as arsenic, did not increase PAH bioactivating enzymes and did not therefore increase carcinogenic potential (Vakharia et al., 2001). While this study does not demonstrate that arsenite affected the induction of PAH activating P450s such as CYP1A1 it does suggest that arsenic may have some role in regulation of CYP1A1 induction (Vakharia et al., 2001). Another possibility for the lack of definitive results is that other pathways and mechanisms are responsible for the apparent increase in carcinogenicity seen in some metal/PAH interactions.

1.4 Methods of detecting DNA damage

Various methods have been used to directly study adducts formed from exposure to BPDE. Measuring human exposure to potentially harmful elements in the environment requires sensitive techniques. These exposures, with the exception of some occupational exposure, are generally low concentration exposures and are in most cases outside of the detection limits of many analytical methods. One of the most sensitive methods for detection of DNA damage is the ^{32}P-postlabeling method (Beach et al. 1992; Baan et al., 1997; Li et al., 2001); this radioactive method allows the detection of one adduct in 10^9 unmodified nucleotides in microgram amounts of DNA. While the ^{32}P-postlabeling method has a very high sensitivity it also has some important disadvantages. Firstly, radioactive materials require special handling and disposal. In addition to this, the ^{32}P-postlabeling method is time consuming and does not provide information about the identity of the adduct. Another method commonly used to measure DNA damage is the comet assay, or single cell gel electrophoresis This method also displays high sensitivity but is most often used to analyze DNA strand breaks. GC-MS (gas chromatography with mass spectrometry) requires enzymatic digestion and chemical derivatization that through oxidative damage may produce artifacts (Goldman et al., 2001). Many others have used other methods to detect and analyze DNA damage (Fang et al., 2001; Pfeifer et al., 1998; Galati et al., 2001). CE-LIF is very sensitive, with detection limits of 10^{-21}, the ability to analyze nanomolar sample concentration (Xing et al., 1999) in a very short analysis time (Tan et al., 2001). The results presented in this section were obtained in sample analyses of 4 minutes or less.

1.5 Objectives

The objectives of this research were to investigate the effects of BPDE and arsenic both alone and simultaneously on A549 genomic DNA using capillary electrophoresis-laser induced fluorescence (CE-LIF). This research involves both development of assays for BPDE-DNA adduct analysis and the application of these assays to study the induction and repair of BPDE-DNA adducts.

The first set of studies described in Chapter 2 involve an assessment of necessary experimental parameters to satisfactorily separate and detect BPDE-DNA adducts. A synthetic oligomer containing a single BPDE adduct is used for this purpose. Capillary length, field strength, and buffer ionic strength are among some parameters investigated and discussed. Chapter 2 also presents results from CE-LIF competitive assays using both genomic DNA and 16 and 90 base pair oligonucleotides with BPDE adducts.

The second set of studies described in Chapter 3 demonstrate the effects of arsenite and BPDE on A549 cellular DNA. Damage and repair of genomic A549 DNA is investigated using a CE-LIF noncompetitive assay. A discussion of the results in the context of current research is included in this chapter also.

Chapter 2. Determination of experimental parameters with BPDE-DNA damage standard

2.1 Introduction

Measuring human exposure to potentially harmful elements in the environment requires sensitive techniques. These exposures, with the exception of some occupational exposure, are generally low concentration exposures and are in most cases outside of the detection limits of many analytical methods. The fluorescent probe of DNA damage developed by Carnelley et al. (2001) in conjunction with the development of the ultra-sensitive assay method of CE-LIF by Le et al. (1998) is used in this section to determine some appropriate experimental parameters for future analysis of BPDE-DNA adducts. Using the prepared BPDE 90-mer allows investigation into parameters such as buffer concentration and denaturation time for example.

2.2 Materials and Methods

2.2.1 Instrumentation

These samples were analyzed on a CE-LIF system using a sheath flow cuvette 2.0 cm long, with 0.9mm thick walls, and a 200 x 200μm inner bore (NSG Precision Cells, Famingdale, NY, USA). The fused silica capillary, 29cm x 20μm I.D x 150μm O.D., (Polymicro Technologies, Phoenix, AZ, USA). The injection end, was connected to a power supply set at positive polarity and was grounded on the other end and was held in place inside the sheath-flow cuvette.

Figure 4 shows a general schematic of the CE-LIF systems used in experiments in Chapters 2 and 3. Analysis of BPDE and control DNA, and the BPDE oligomer was performed by a capillary electrophoresis laser-induced fluorescence (CE-LIF) system built in this laboratory. A CZE1000R, Spellman High Voltage Electronics power supply was used to provide electrophoretic potential. A LabVIEW (National Instruments, Austin, Texas) program run on a Macintosh computer controlled separation conditions including sample injection time and voltage and also separation voltage and sample run time.

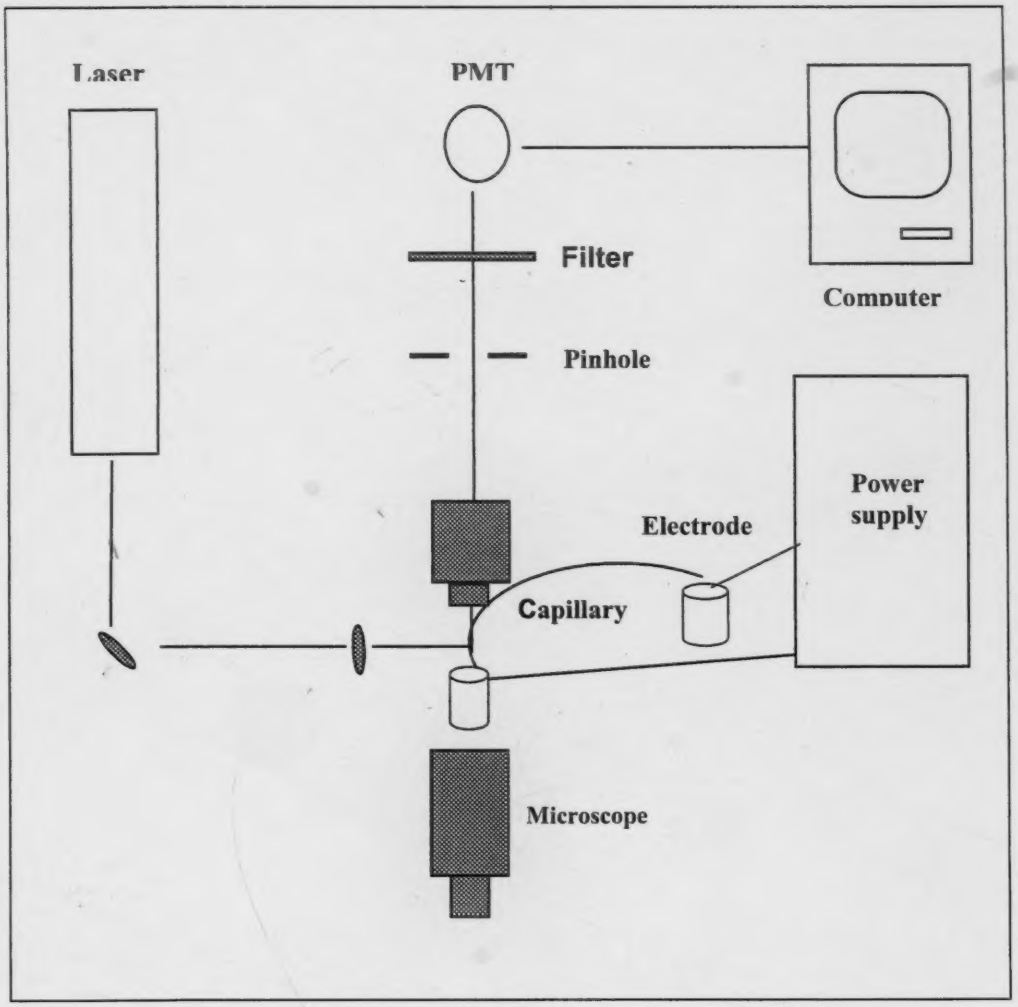

Figure 4. Schematic of CE-LIF instrumentation.

Detection was provided by a laser-induced fluorescence system that contained custom-made and commercial components and was built on an optical table. The laser source was a 1.0mW, green helium-neon laser (Melles Griot, Irvine, California) with an excitation wavelength of 543.5 nm. An overview of the optical path follows. The helium-neon laser was focused on the capillary through a 6.3x microscope objective, 90° to the laser was a high numerical aperture objective that collected the fluorescence signal (0.7 numerical aperture, 60x, Universe Kogaku, Oyster Bay, New York). The signal then passed through a changeable diameter pinhole that was adjusted to allow the minimum amount of background light. Following this, the signal passed through a bandpass filter that eliminated laser light scatter; a photomultiplier tube then detected the fluorescent light. Finally, the signal was recorded by a Macintosh computer running LabVIEW software and with a PCI data acquisition board.

The system was aligned every day prior to use. A microscope was used to visualize the alignment of the laser beam with respect to the collection optics that were represented by a light emitting diode (LED) positioned behind the adjustable pinhole. Alignment was accomplished by fixing the position of the collection assembly, then by adjusting the capillary and laser-focusing objective with the X-Y-Z translation stages. Alignment could be optimized by adjustment of the angle of the fluorescence-collecting objective lens and the position of the collection assembly.

2.2.2 Synthetic oligonucleotide sample preparation

A detailed protocol of preparation of the BPDE-DNA 90-mer is available in a publication by Carnelley et al., (2001). A summary of the procedure from Tan et al., (2001) follows. A 16-mer oligonucleotide, 5'-CCCATTATGCATAACC-3', was treated with BPDE at a molar ratio of 1:5 (oligonucleotide:BPDE). The oligonucleotide was reconstituted in a 20mM phosphate buffer-1.5% triethylamine at pH 11. A BPDE solution was added to the oligonucleotide to a final BPDE concentration of 270μM. The mixture was incubated at room temperature, overnight in the dark with gentle shaking. Purification of the BPDE-modified oligonucleotide was accomplished in two separate rounds of HPLC elution using a preparative C_{18} column. (250 x 10mm, 5μM particle size, Phenomenex, Torrance, California, USA). The first round involved isocratic elution using 70% methanol and 30% of 20mM phosphate, pH 7; this was used to purify the BPDE-oligonucleotide by separating the oligonucleotides from free BPDE. Eluent containing the BPDE-oligonucleotide and unmodified oligonucleotide was freeze-dried and then separated with a second round of HPLC using a gradient elution of methanol-20mM phosphate at pH 7. The purified BPDE-oligonucleotide then underwent ligation to 5 other nucleotides to produce a BPDE-DNA duplex of 90 base pairs. The duplex was purified using a 7.5% native polyacrylamide gel, and subsequently subjected to UV and fluorescence to measure DNA concentration and to detect the presence of the BPDE group on the duplex.

Figure 5 is a schematic diagram of a synthetically constructed 90 base-pair oligonucleotide duplex. The duplex is constructed by ligation of 6 smaller oligonucleotides, one of which contains an adduct resulting from exposure to 270μM

BPDE. Only one strand of the duplex contains the BPDE-DNA adduct and is labeled at its 5' end with a tetramethylrhodamine (TMR) dye label. This synthetic 90-mer was used as a probe in a competitive assay to detect the levels on BPDE-DNA damage in an A549 cell line.

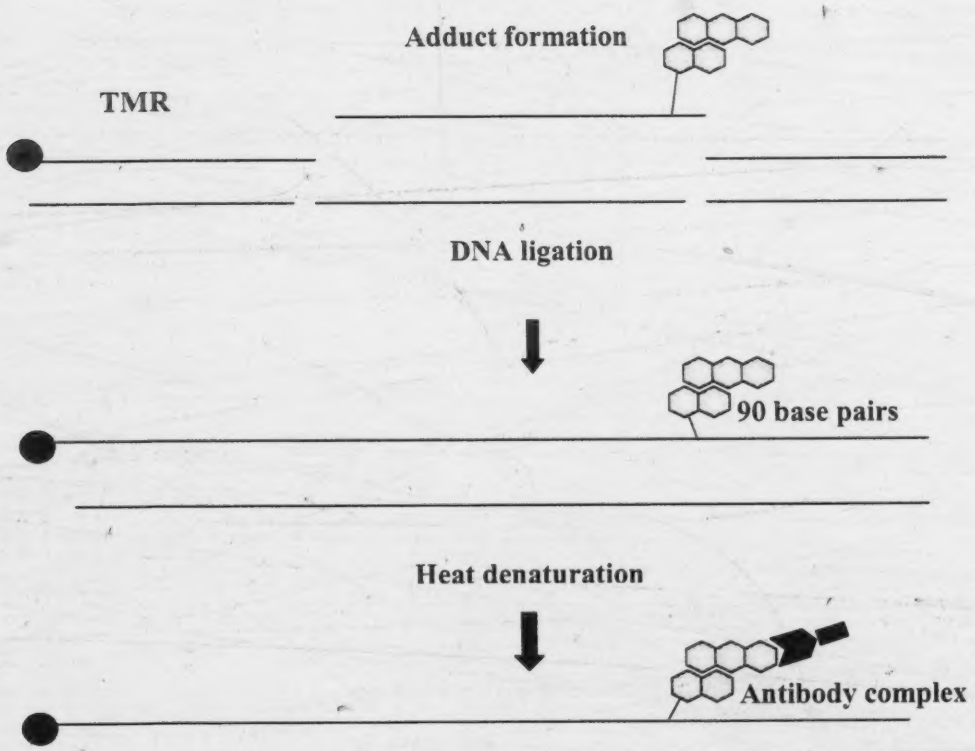

Figure 5. Components of a 90 base-pair oligonucleotide duplex BPDE-DNA standard. The duplex consists of 6 smaller oligonucleotides joined through ligation. One of the strands contains a BPDE-DNA adduct and is labeled at the 5' end with the fluorescent dye TMR. After heat denaturation the BPDE lesion is readily accessible to a BPDE-specific antibody. (Adapted from Carnelly et al., 2001).

2.2.3 Analysis of BPDE standard

Samples were injected electrokinetically with an injection voltage of 10000 V for 5-10 seconds and running voltage was 20000 V. The capillary was washed every 3-5 runs with 0.2 M NaOH and 1x Tris glycine for 5 minutes each. Data was analyzed using Igor Pro software (version 3.1, WaveMetrics Inc., Lake Oswego, Oregon).

2.3 Results and Discussion

An objective of these experiments was to develop a competitive assay using the fluorescently labeled oligonucleotide as a probe. These experiments involve the addition of both the standard TMR-labeled probe and a BPDE-specific antibody (8E11) to an A549 DNA sample containing an unknown amount of BPDE damage/adducts. The A549 DNA is not labeled and adducts in these samples compete with labeled probe adducts for the BPDE-specific antibody. The 8E11 antibody is bivalent and the antibody-BPDE adduct complex will result in various products. Firstly, the A549 BPDE-DNA adduct may produce complexes consisting of one or two adducts bound to an antibody. Unbound adduct may also be a sample component. The antibody may also bind to one or two probes and unbound 90-mer may also exist in the sample. Separation of these various products with capillary electrophoresis allows an indirect quantitation of the A549 BPDE-DNA adducts.

2.3.1 Capillary Length

Capillary length is an important parameter to help ensure stable and detectable complex formation in a reasonable period of time. Figure 6 shows that 30 and 35 centimetres resulted in short analysis time with no apparent loss of resolution. Longer capillaries may allow the complex time to disassociate and resolution will therefore be poor. As is evident in Figure 6, the antibody-90-mer complex peak (denoted as peak 1) decreases with capillary length from 30 to 60 m. At 60 cm, capillary shows very little of the complex peak but the free oligomer peak is quite large; this result is likely due to disassociation of the complex peak in the capillary. Longer capillaries may also allow more time for sample adsorption to capillary walls.

2.3.2 Buffer Ionic Strength

Ionic strength is another important parameter that may affect complexation between adduct and antibody. Tris glycine buffers of various ionic strengths were used to determine the most suitable ionic strength for the separation of BPDE 90-mers. Figure 7 shows a deterioration of separation at 0.25 x Tris glycine. This may be due to greatly increased EOF (electroosmotic flow) which did not allow oligomers to be adequately resolved or also perhaps due to sample adsorption to the capillary wall At 0.5 x to 3x, separation of 90-mers was maintained and the peak areas of bound and unbound adducts increased from 3.0 x up to 0.5 x Tris glycine. Increased ionic strength results in decreased EOF; this is demonstrated by the up to 4 minutes required for separation of oligomers with 3 x Tris glycine buffer in contrast to the approximately 2 minute separation time accomplished with 0.5 x Tris glycine. In addition to this, the ratio of bound to unbound oligomers has increased from 3x to 0.5 x Tris glycine buffer.

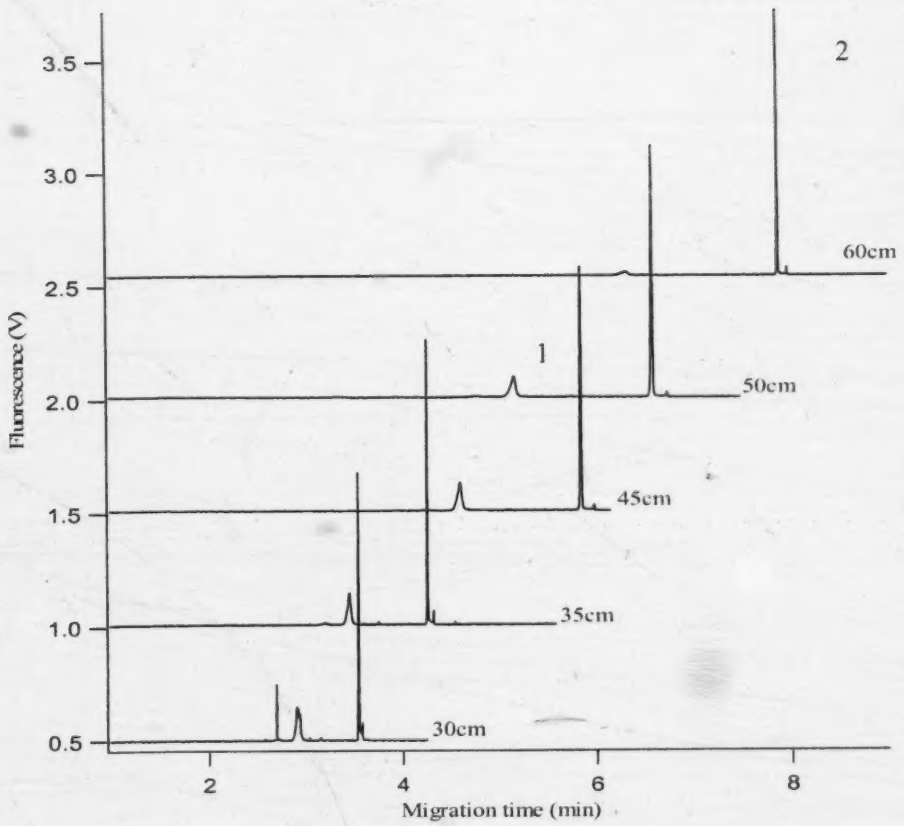

Figure 6. Effect of capillary length on separation of the BPDE 90-mer-antibody complex (peak 1) and the unbound 90-mer (peak 2). A capillary was cut from 60cm to 30cm.

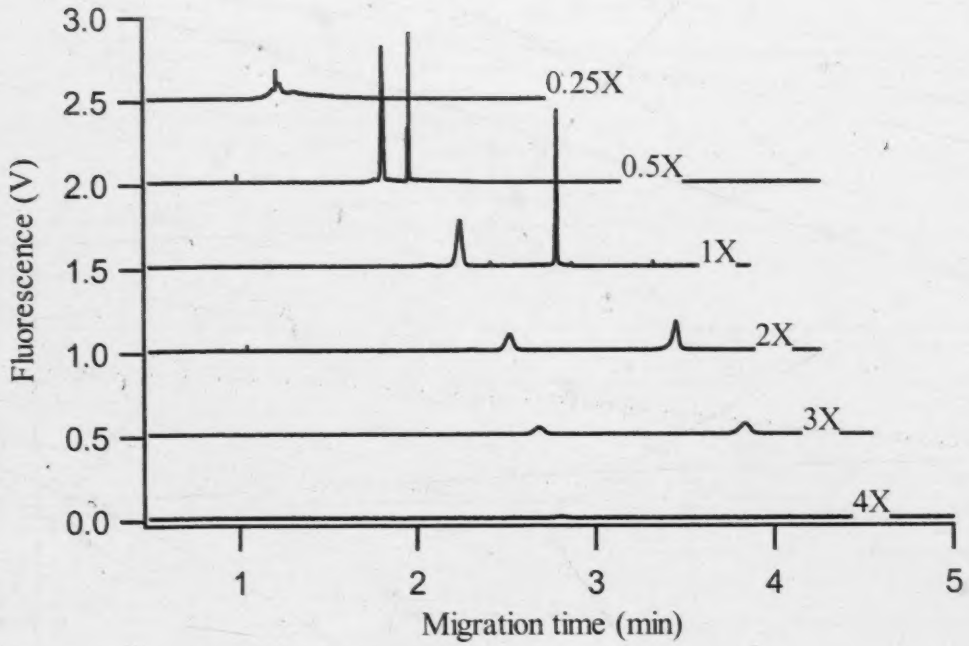

Figure 7. Effect of Tris glycine buffer concentration on separation. A serial dilution of a 10x Tris glycine stock solution was performed to give 3x, 2x, 1x, 0.5x and 0.25x Tris glycine.

2.3.2 Incubation time and temperature

Antibody binding to DNA was observed by capillary electrophoresis at temperatures as low as 0° Celsius and with incubation times as short as one minute. Room temperature incubation for 5 to 10 minutes was selected as the conditions giving best results. Disassociation of sample complex peak could be seen after 20 minutes at room temperature and 30 minute incubation.

2.3.3 Denaturation of DNA

Binding of 8E11 gave better results with the DNA adduct on a single strand than binding with the double stranded DNA adduct. Because of this, a denaturation step was incorporated into the procedure. Formamide was added to the incubation buffer to ensure that reannealing of complementary oligo strands did not occur during the electrophoretic process. Figure 8 shows the effect of formamide on the stability of the complex. Formamide did not affect the complex up to a concentration of 12.5% (v/ v) and also improved the resolution between BPDE 90-mers that were bound to antibody and 90-mers that were not bound (bound and unbound adducts). At 82.5% formamide, bound and unbound adduct peaks appear distorted. Higher peak intensities in Figure 8(a) may indicate a decrease in ionic strength due to increased formamide and thus larger injection volume.

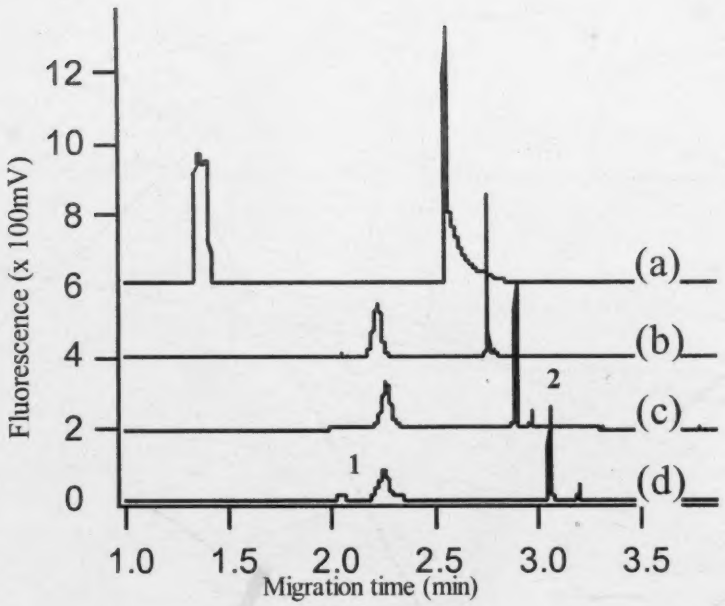

Figure 8. Effect of formamide on BPDE-90-mer-antibody complex stability. Following is the concentration of formamide (v/ v) in incubation buffers (a) 82.5% (b) 12.5% (c) 5% (d) 2.5%. Peak 1 is the complex of BPDE-90-mer standard and 8E11 antibody and peak 2 is the unbound BPDE-90-mer

2.3.4 Electrophoresis field strength

Determination of the best field strength for these experiments is essential to achieving optimal resolution. Using a 30 cm capillary, field strength was adjusted from 25 kV to 5 kV (see Figure 9). The 25 kV CE (running) voltage appeared to provide the best resolution in approximately 2 minutes. The field strength at 25 kV, was 830 V/cm with field strength being proportional to electroosmotic flow. As field strength and EOF increase, so do problems associated with excess joule heating. Joule heating can result in the increase of band broadening leading to lower resolution and also local temperature changes inside the capillary which can in turn lead to local viscosity changes. In general, joule heating can result when a substantial temperature gradient arises between the center of the capillary and the capillary walls. To remedy this problem, the selection of a narrow bore capillary (small internal radius) can allow a smaller internal gradient and heat dissipation through the larger surface area. The electrical field experiments showed no apparent sample degradation due to joule heating so the selected electric field and buffer ionic strength are suitable.

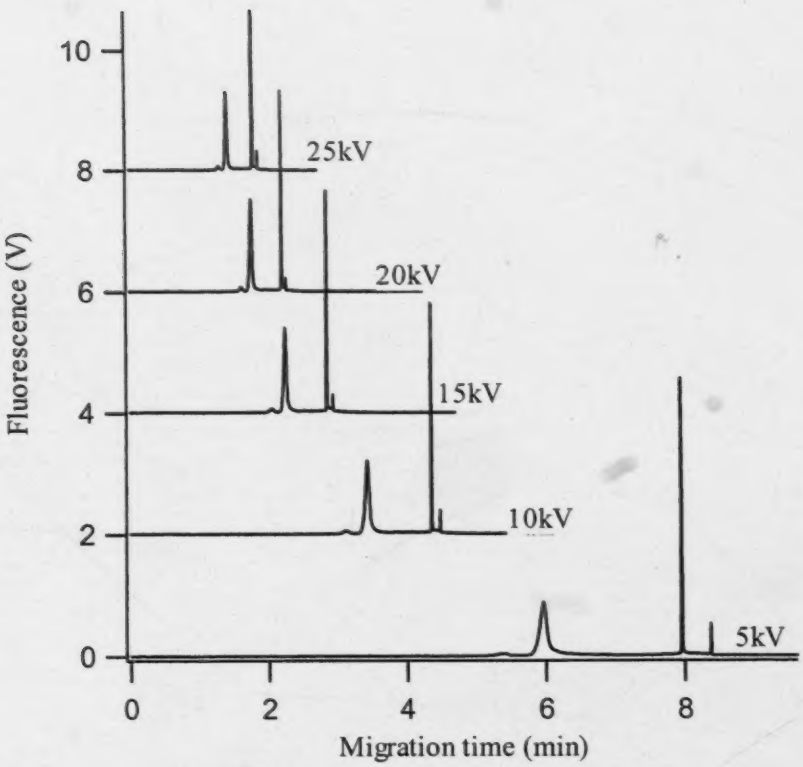

Figure 9. Effect of field strength on separation of bound and unbound BPDE 90-mers. CE voltage was increased from 5 to 25 kV. Analysis time decreased with increased running voltage while resolution improved.

2.3.5 Competitive assay

In this type of assay, a fluorescent probe competes with the target analyte for the limiting amounts of antibody. In this study, a TMR-labeled 16-mer and a TMR-labeled 90-mer, each containing a single BPDE adduct, were used as the fluorescent probe. Mouse monoclonal antibody (8E11) was used to recognize the BPDE-DNA adducts.

It is important to note that 8E11, the antibody used in the noncompetitive as well as competitive assays, is bidentate; this means that that each antibody may bind with one or two antigen molecules. Therefore we can expect to see an unbound oligomer peak as well as complex peaks resulting from a 1:2 or 1:1 ratio of antibody to BPDE antigen (90-mer, 16-mer or in A549 DNA).

Figure 10 shows the results for a competitive assay using a TMR-labeled BPDE-16-mer and an unlabelled BPDE-16-mer with 2 μg/ L. As unlabeled 16-mer concentrations are increased, the 1:1 (peak 1) and then 1:2 (peak 2) complex peaks decrease. This may be due to the presence of excess amounts of 16-mer, favoring the formation of the 1:2 complex.

Figure 11 shows a competitive assay using 16-mer (unlabeled) and 90-mer (TMR-labeled) BPDE damaged probes. As is evident from the results antibody binding can be accomplished in the presence of the 90-mer; these results are similar to those in Figure 10.

The 90-mer was then used as a probe in a competitive assay for BPDE-DNA adducts in A549 cells treated with 9.4-300 μM BPDE. Figure 12 shows that as BPDE concentration increases more 8E11 antibody is bound to the unlabeled BPDE-DNA adducts present in the A549 DNA, and the signal from the unbound 90-mer probe increases. As BPDE concentration increases DNA adduct formation in A549 cells also increases.

In conclusion, increased concentration of BPDE results in increased damage to A549 DNA. Increased adduct formation is shown by increased antibody binding to the damaged site. This competitive assay in combination with capillary electrophoresis is an effective method to demonstrate the effects of
BPDE on A549 DNA.

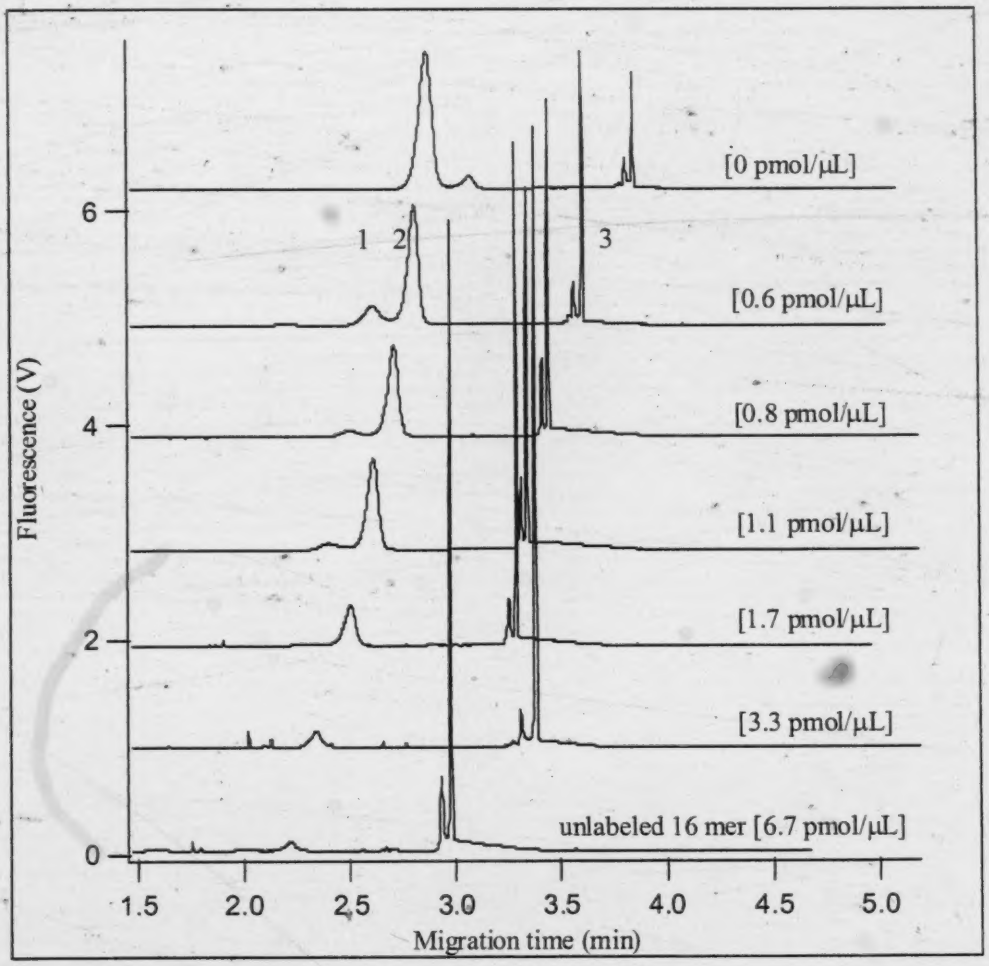

Figure 10. Electropherograms showing results of competitive assay using unlabeled BPDE 16-mer and the TMR-labeled BPDE 16-mer as probes. Peaks 1 and 2 represent the 1:1 and 1:2 ratio of primary antibody to adduct(s). Peak 3 is the unbound TMR-labeled 16-mer.

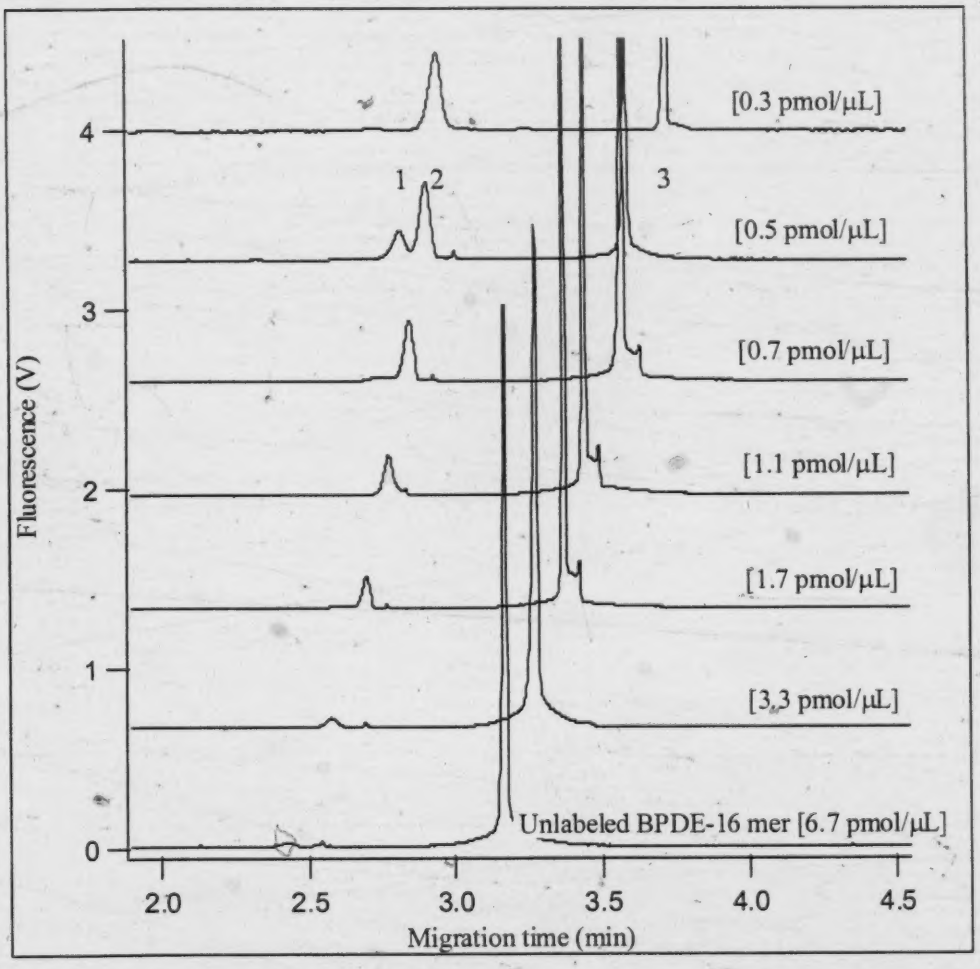

Figure 11. Electropherograms from competitive assay using unlabeled BPDE 16-mer and TMR-labeled BPDE 90-mer as a probe. Peaks 1 and 2 represent the 1:1 and 1:2 ratio of primary antibody to adduct(s). Peak 3 is the unbound TMR labeled 90-mer.

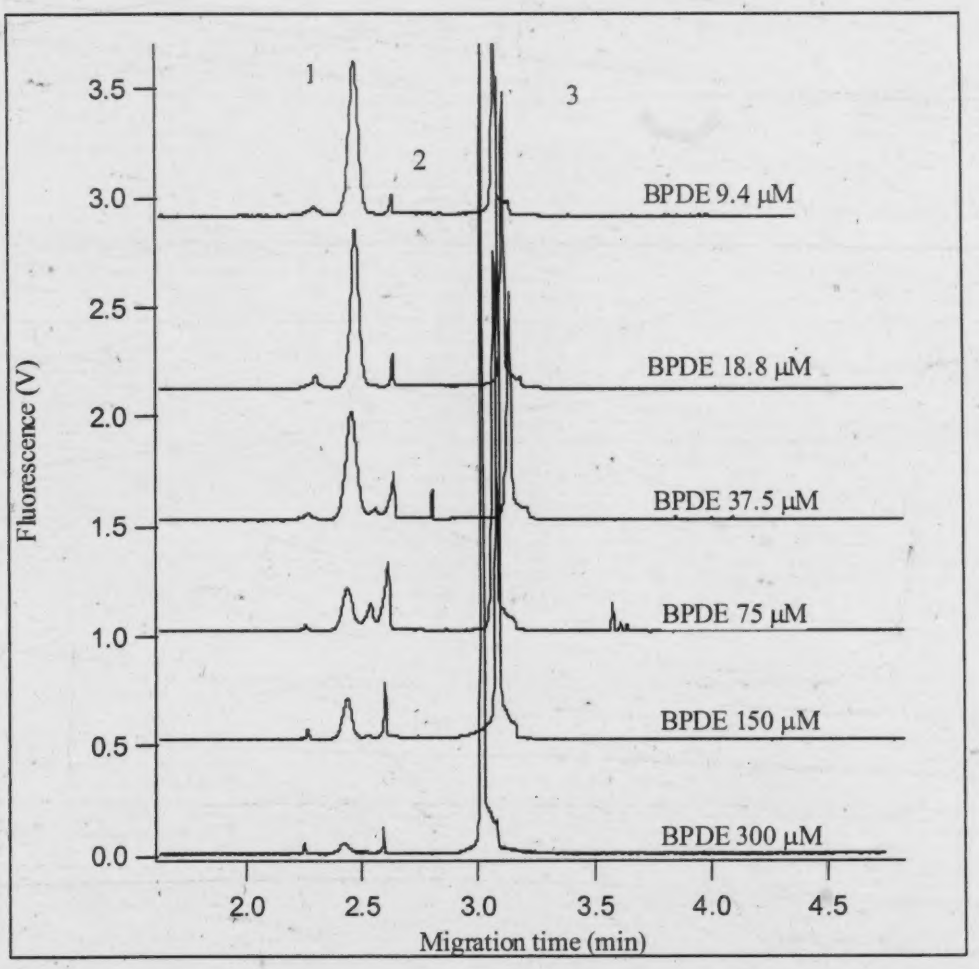

Figure 12. Competitive assays electropherograms for A549 cells treated with 0-300 μM BPDE using TMR-lableled BPDE 90-mer as a probe. Peaks 1 and 2 represent the 1:1 and 1:2 ratio of primary antibody to adduct(s). Peak 3 is the unbound TMR labeled 16-mer.

Chapter 3. Effects of BPDE and arsenite in the induction and repair of BPDE-DNA adducts in A549 cells.

3.1 Introduction

The competitive assays in Chapter 2 used one antibody and a labeled probe to determine unlabeled DNA adduct in cell samples. The following experiments use noncompetitive assays that use two antibodies, one of which is labeled fluorescently secondary antibody and the other is unlabeled primary antibody 8E11 that is specific for BPDE damage will be bound by a fluorescently labeled (secondary) anti-mouse antibody, Alexa Fluor 546. Noncompetitive assays measure damage more directly than competitive assays; an increase in the fluorescent signal from the tertiary complex (secondary antibody-primary antibody-DNA adducts) is an indication of increased damage.

BPDE has been recognized to be a human carcinogen and much work has been done to demonstrate the effects of BPDE in cells *in vitro* and *in vivo*. (Galati et al., 2001; Li et al., 2001; Venkatachalam et al., 1995; Wang et al, 1996; Maier et al., 2002; and many others). DNA damage resulting from BPDE can be viewed as the sum of metabolic activation and detoxification as well as repair (Wang et al., 1996). It is important to note that this damage will vary from individual to individual and among cell lines. The effects of BPDE such as DNA damage and cytotoxicity have been demonstrated in other experiments; it cannot be assumed though that these results are directly applicable to A549 cells.

The focus of this chapter is the effect of a mixture of BPDE and arsenite on the damage and repair of A549 DNA. Experiments using only one carcinogen may provide insight into the carcinogen's effect on human health, but most people are exposed to a mixture of various environmental components and some of these are carcinogens in low concentrations. Investigations into, for example, mixtures such as arsenite and BPDE may provide insight into human exposures. For example, PAHs in tobacco smoke are thought to be too low to induce carcinogenesis by themselves but the presence of effective concentrations of carcinogens and promoters allows the development of tumors (Rubin, 2001). Likewise, unrefined coal tar induces tumors that appear earlier, are larger, and produce more malignant tumors than tumors induced by concentrations of pure PAHs far higher than the PAH concentration found in coal tar; these results suggest that synergistic interactions may promote carcinogenesis (Rubin, 2001).

3.2 Materials and methods

3.2.1 Reagents

(±)-anti-r-7,t-8-dihydroxy-t-9,10-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (BPDE) was provided by the National Cancer Institute Chemical Carcinogen Reference Standard Repository at the Midwest Research Institute in Kansas City, Missouri. BD Pharmingen, San Diego, California, supplied the primary antibody 8E11. The secondary (goat anti-mouse antibody) Alexa Fluor 546 was obtained from Molecular Probes, Eugene, Oregon. Dimethyl sulfoxide (DMSO) was provided by Sigma. DNA was extracted with DNAzol Reagent purchased from Life Technologies, GibcoBRL.

Dulbecco's Modified Eagle's Medium/Ham's F12 (DMEM/F12) cell media were purchased from Gibco. Fisher supplied sodium hydroxide and glycine; Tris was purchased from GibcoBRL. Aldrich Chemical Company provided the arsenic standard.

3.2.2 Instrumentation

These experiments also used CE-LIF instrumentation similar to that illustrated in Chapter 2, (Figure 4) except that the capillary was not held in place by a cuvette. These experiments used fused silica capillaries (Polymicro Technologies, Phoenix, Arizona) with an internal diameter of 21 μm and an o.d. of 150 μm; total capillary length was 36 cm and an effective length of 31 cm. In general, the injection end of the capillary and an electrode connected to the power supply, which was set at positive polarity, were placed into the sample and/or running buffer while the other end of the capillary was held securely in place by a grounded holder and drained into a waste reservoir.

3.2.3 Analysis with CE-LIF

Samples were injected electrokinetically into the capillary by applying an injection voltage of 10000 volts (V) for ten seconds. The samples were separated with a running voltage of 20000 V. The running buffer was 1x Tris glycine (25 mM Tris, 250 mM glycine), pH 8.3. The capillary was washed after every three sample runs for 5 minutes each with 0.2 M NaOH and followed by 1x Tris glycine injected electrokinetically and run at 20000 V. Overnight the capillary was flushed with HPLC-grade water applied by a syringe. Data was analyzed using Igor Pro software (version 3.1, WaveMetrics Inc., Lake Oswego, Oregon).

3.2.4 Preparation of A549 genomic DNA samples

3.2.4.1 Cell treatment

Genomic DNA used in analysis was extracted from A549 human lung carcinoma cells with the help of Jane Lee and Nan Mei and facilities at the Cross Cancer Institute. In order to seed approximately 1 x 10^5 cells per plate, 30μL of cell suspension were transferred from 25cm^2 canted neck tissue culture flasks (culture started by Jane Lee or Nan Mei) that were stored in the CO_2 incubator to tissue culture plates (60 x 15mm Falcon 35) containing 2ml of DMEM/F12 media with 10% fetal bovine serum (FBS). Seed enough plates to ensure an adequate number for various treatments, duplicates, control and also include two plates for cell counting. Incubation conditions were 95% humidity, 15% carbon dioxide (CO_2), and 37.4 °C. When cells had grown to a monolayer, media was removed from plates and plates were washed 2x with 1x

phosphate buffered saline (PBS) which was then replaced with 2ml media containing BPDE or BPDE and arsenic III and incubate for 2 or 4 hours.

For repair experiments: A549 cells were pretreated with arsenic III for a 24 hour period before the four-hour exposure to BPDE and arsenic III. Immediately prior to use, prepare a stock solution of BPDE dissolved in DMSO (to give 3mM), store in a dark bottle, and protect from light. For arsenic repair experiments: dilute arsenic III solution to required concentration in cell media.

For dose-response experiments: label bottles with appropriate concentrations of BPDE and dilute BPDE stock solution (3mM) with appropriate volumes of DMEM/F12 media. Remove plates from incubator and discard media. Plates were washed twice with PBS and 2ml of DMEM/F12 with each of 1, 3, 5, 7, 10uM BPDE was added to each plate. Each plate was labeled with the concentration of BPDE it received. Cells were then placed into the incubator for 2 hours. Following 2 hour incubation, media was discarded, plates were washed 2x with PBS and DNA was extracted.

For BPDE repair experiments: cover a centrifuge tube (or larger bottle depending on volume required) with tinfoil and label and add required volume of media; dilute BPDE stock solution in this media to required concentration. For these experiments cells were incubated with 2, 6, 20, and 40 μM of BPDE for 2 hours. Following incubation genomic DNA was extracted.

3.2.4.2 DNA extraction

After 2 or 4-hour incubation period, media was removed from plates and plates were rinsed 2x with PBS (2mL per wash). PBS was removed and 1ml DNAzol reagent was added to plates. The plates were left for a few minutes and when solution turned gluey, lysate was pipetted off into labeled test tubes (cut off tip to prevent shearing of DNA). Into each tube 0.5ml of ice cold 99.9% ethanol was added. Suspension was left at room temperature for five minutes and then inverted a few times. DNA appeared as a white precipitate. If no DNA was visible mixture was centrifuged for 15 minutes at 1000 rpm. 1.5ml eppendorf tubes were labeled and 0.5ml of 70% cold ethanol was added to each tube. DNA was then transferred into Eppendorf tubes by wrapping the DNA around a pipette tip. Tubes were inverted a few times and ethanol was removed. Next, 0.5mL of 95% ethanol was added 2x and tubes were inverted a few times. Ethanol was removed and DNA was allowed to dry until no ethanol was visible. DNA pellet was dissolved in 100μL deionized water and left at room temperature overnight. On the following day, DNA concentration were checked using spectrophotometer (Beckman DU7400). Samples were adjusted to required concentration with 1x Tris glycine (150 μg/mL used for A549 DNA).

3.2.5 DNA sample analysis

To prepare samples for analysis, 0.2 mL tubes were labeled, and 12.5 μL of DNA solution and 2.5 μL of 1x Tris glycine were pipetted into the tubes. After 5 minutes of denaturation in a heat block at 95°C and then placed on ice immediately for 10 minutes. 4μL of primary antibody 8E11 (purified mouse anti-benzo(a)pyrene diol-epoxide monoclonal antibody) was pipetted from 8E11 stock solution. Stock solution was prepared with 23 μL, 1x Tris glycine + 1 μL 8E11 (2 mg/ml) + 1μL Human IgG (diluted to 1μg/ml) into the sample tube to give a final concentration of 20 μg/mL. Sample was removed from ice and incubated at room temperature for 30 minutes. After 30 minute incubation, 1 μL of Alexa Fluor 546 secondary antibody ($F(ab')_2$ fragment of goat anti-mouse IgG) was added to sample to give a final concentration of 2 μg/ ml. Sample was stored on ice for at least 5 minutes (to allow secondary antibody binding) prior to running on CE-LIF system. Sample was analyzed as soon as possible after incubation with secondary antibody.

3.3 Results and discussion

3.3.1 Dose response experiment

Capillary electrophoresis was used to measure damage in A549 DNA resulting from 2 hour exposures to 0 (control), 1, 3, 5, 7,and 10 μM BPDE concentrations. Figure 14 shows the dose-response curve resulting from this experiment. Each data point is the complex peak area measured at each dose. This graph shows that as BPDE concentration

increases damage to the cellular DNA also increases. At 5, 7, and 10 μM, the rate of lesion formation appears to have decreased.

The dose-response experiment (Figure 13) gives an indication of the cellular response to varied concentrations of BPDE. Figure 13 shows what appears to be a response plateau at the 5, 7, and 10 μM concentrations of BPDE. This response may indicate that the above-mentioned equilibrium between metabolism and detoxification has reached constant rate or instead may represent cytotoxic effects and a loss of ability to repair or detoxify.

In response to this uncertainty, low doses of BPDE that have been shown by other researchers to be noncytotoxic working doses were chosen. Wang et al., (1996) treated in vitro lymphocytes with 4 μM BPDE to avoid cytotoxic effects that would result from higher dose exposures. Li et al., (2001) found that exposing peripheral lymphocytes to concentrations of BPDE up to 10 μM gave greatly varied relative adduct levels above 4 μM. Influenced by these results, experiments included in this paper, with one exception, have used relatively low dose exposures.

3.3.2 Time-response of A549 genomic DNA to 1uM BPDE.

BPDE lesions appear to decrease over time in A549 DNA allowing further investigation into the formation of DNA damage in response to both 1 uM BPDE and 100 μg/L of arsenic III. Figure 14 shows a graph of peak areas obtained from the exposure of A549 cells to 1 μM BPDE for 4 hours followed by incubation in BPDE-free cell media for 0, 2, 4, 16, and 24 hours. DNA damage, demonstrated by peak area, appeared to be the greatest at 4 hours. 0, 2, 16, and 24 hour exposures demonstrate smaller complex peaks. At 24 hours complex peak areas appear close to areas obtained from the control and 2 hour exposure samples.

In order to remain healthy, an organism must be able to repair damage from many sources. The capacity of cells to repair DNA damage may depend on many features. The concentrations of BPDE and arsenite as well as type of cell line used are an important factor in the cellular response. Figure 14 shows the peak areas of A549 cells exposed to 1 μM BPDE over 24 hours. These results do not indicate that adducts are greatest at four hours or that adducts decrease immediately after 4 hours but that out of the concentrations of BPDE used, cells exposed for 4 hours showed the greatest number of adducts with CE-LIF analysis. Kuljukka-Rabb et al. (2001) exposed MCF-7 cells to 2.5 μM benzo(a)pyrene for up to 48 hours and noticed a decrease in adducts only after 12 hours. While the experiments of Kuljukka-Rabb et al., (2001) used benzo(a)pyrene and not BPDE as well as the MCF-7 cell line and not A549, the trend of increase in adducts and then a decrease is similar to my results. A549 are particularly suited to these

experiments because BPDE has been found to be a poor carcinogen on mouse skin unlike its parent compound B(a)P but has been a very effective in resulting in lung adenocarcinomas in newborn mice (Rubin, 2001).

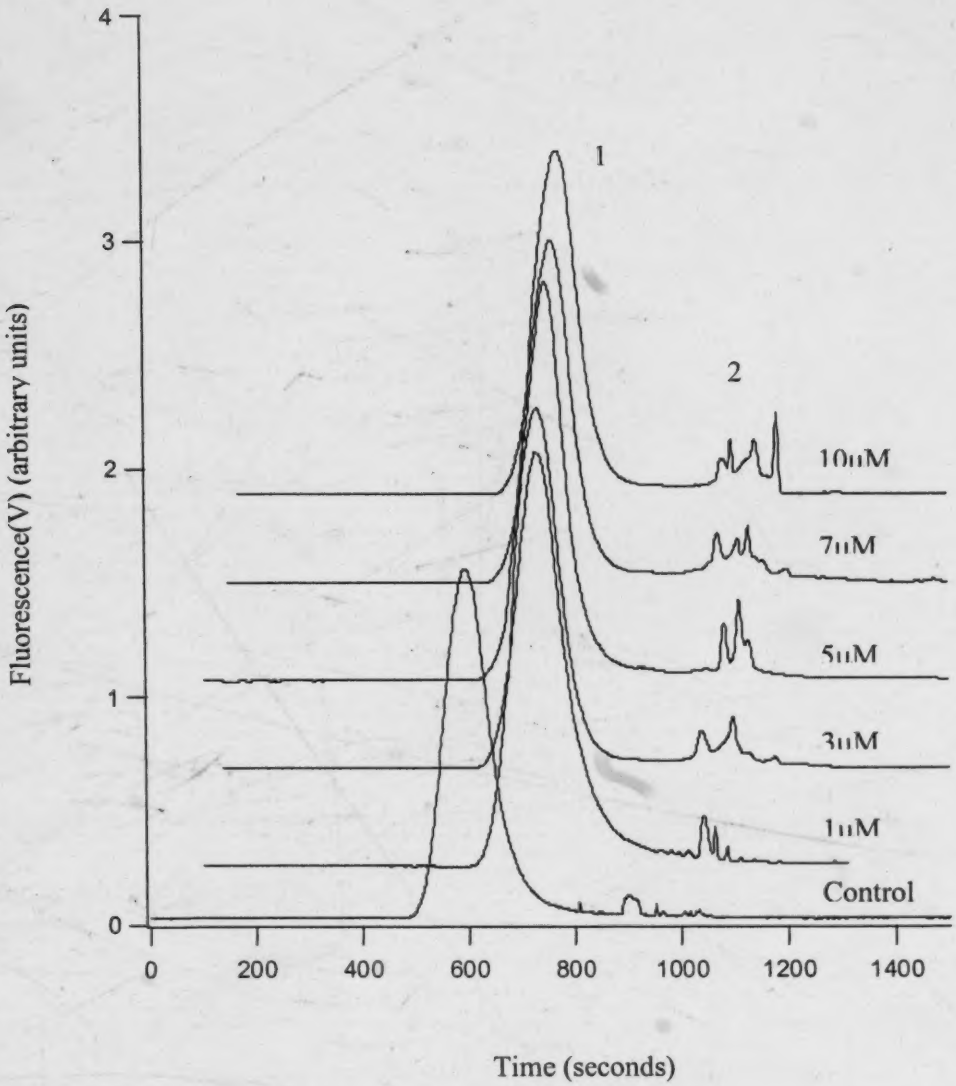

Figure 13. Dose-response electropherograms from A549 cells treated with 0-10 μM BPDE for 2 hours. Peak 1 is the free secondary antibody, A546 and peak 2 is the complex peak.

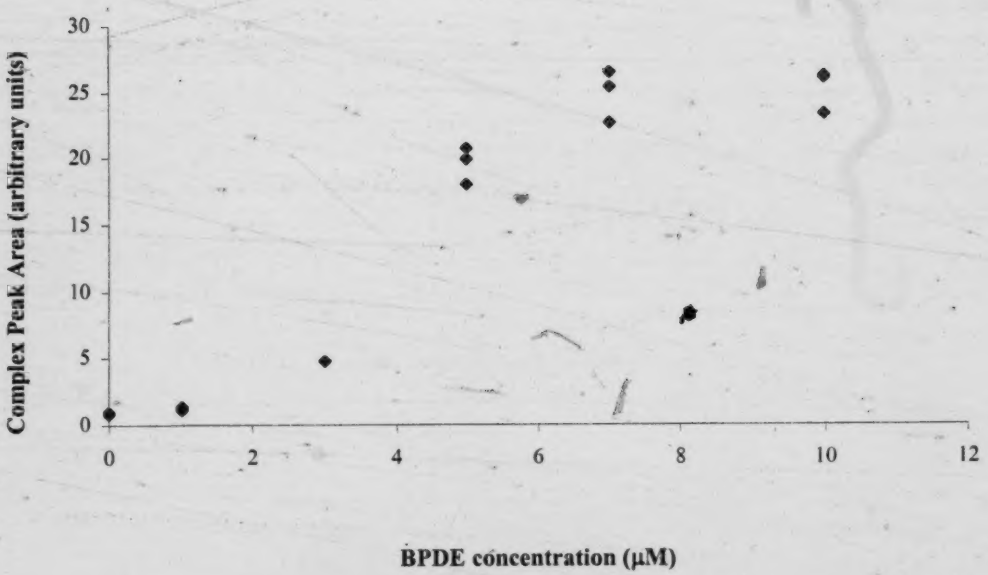

Figure 14. Dose-response curve for A549 DNA treated with 0 (Control), 1, 3, 5, 7, and 10 μM BPDE for 2 hours. Electropherogram shows complex peak areas for sample triplicate runs.

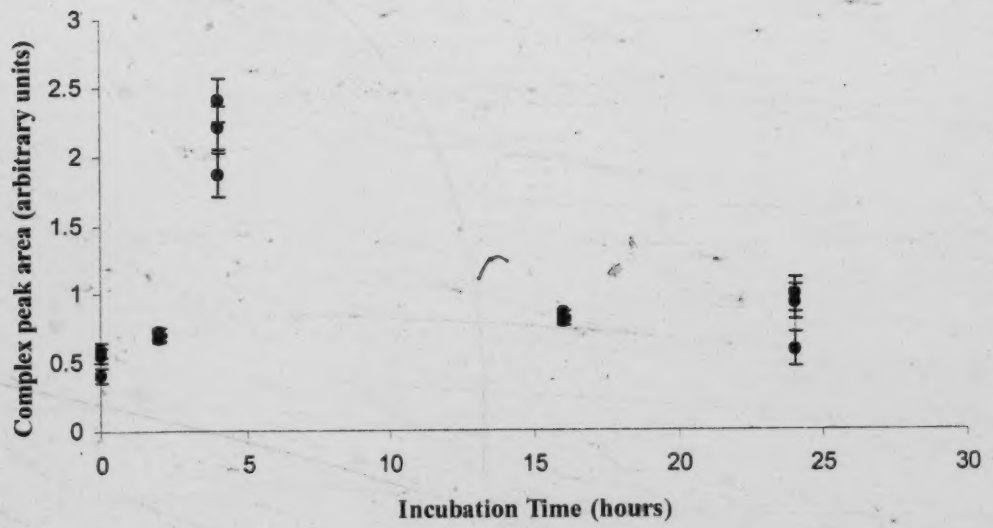

Figure 15. Complex peak areas for A549 cells exposed to 1 uM BPDE for 4 hours and then incubated in BPDE-free media for 0-24 hours. Each time period shows peak areas and error bars for each sample run in triplicate.

3.3.3 Time-response of A549 cells exposed to 1 μM BPDE and 100 μg/L As ^{III}

The repair of adducts may also depend on the initial amount of damage inflicted; in this case, this is the concentration of BPDE and/or arsenite to which cells have been exposed. Table 3 shows that cells exposed to arsenite demonstrated a 10-fold increase in damage at 0 hours than A549 cells exposed to BPDE alone. It is evident that cells exposed to both arsenite and BPDE have received a greater amount of damage but it is not possible to predict from these results how the initial insult affects the rate of repair because repair appears to occur between hour 4 and hour 16. A more in-depth look into the repair that occurs between 4 and 16 hours could determine the effect of the initial exposure concentration. Venkachalam et al., (1995), for example, treated normal human fibroblasts with 0.02 to 4.0 μM BPDE for 30 minutes; cells were then incubated in BPDE-free media for up to 24 hours. As in my experiments using A549 cells, normal fibroblast cells showed adduct removal at 24 hours. Venkatachalam et al. (1995) found that cells receiving all levels of exposure showed adduct repair but those cells that had been exposed to low dose concentrations (0.02 and 0.1 μM) repaired 50% of the damage evident at 0 hours within 4-8 hours while those cells exposed to 2.0 and 4.0 μM (high doses) had repaired only 33 % and 20 % of initial adducts at 24 hours. Keeping in mind that these results were obtained from normal human fibroblasts, it may be possible that A549 cells also remove adducts at a rate dependant on the initial insult.

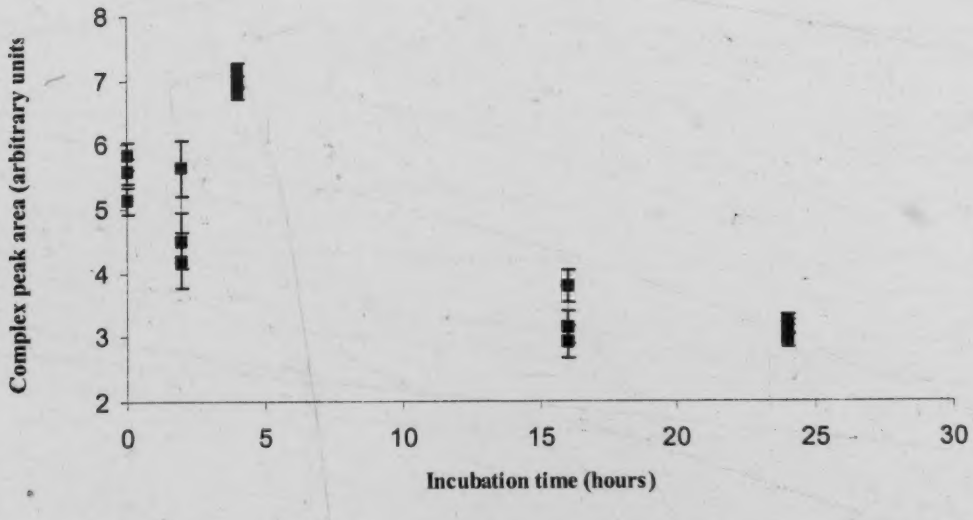

Figure 16. Complex peak area for A549 cells receiving As III pretreatment for 24 hours, 4 hours exposure to 1 μM BPDE + As III (100 μg/L) followed by incubation for 0-24 hours in cell media containing As III only. Each time period shows peak areas and error bars for each sample run in triplicate.

3.3.4 Comparison of A549 DNA cells exposed to 1 μM BPDE and exposed to 1 μM BPDE alone and in combination with 100 μg/L As ^{III}.

Table 3 shows a comparison of complex areas at each time point for both As III + BPDE treated cells and cells treated with BPDE only. At all data points the complex peak areas obtained from BPDE and As III treated cells are greater than areas obtained from cells treated with BPDE only. The greatest difference in complex peak areas is at the 0 hour data point with DNA extracted from A549 cells treated with As (III) and BPDE being 10.6 times greater than the averaged value from A549 cells treated with 1.0 μM BPDE only.

Table 3. Shows complex peak areas for cells exposed to 1 μM BPDE only (in BPDE column) and to 100 μg/L arsenite and 1 μM BPDE for 4 hours and incubated in BPDE-free (BPDE only) or arsenite free (BPDE+ AsIII column) for 0, 2, 4, 16, 24 hours. The ratio of the complex peak areas for BPDE + AsIII treated cells to BPDE only treated cells is shown for each time period.

Incubation Time	0		2		4		16		24	
	Complex Peak Areas									
	BPDE + As III	BPDE	BPDE + As III	BPDE	BPDE + As III	BPDE	BPDE + As III	BPDE	BPDE + As III	BPDE
	5.5908 5.1310 5.8298	0.4129 0.5901 0.5553	5.6350 4.5081 4.1906	0.7330 0.7426 0.5738	6.6840 7.1285 6.8121	2.8055 1.6626 2.407	2.9934 3.1484 3.6832	0.7894 0.8531 0.8055	2.1558 2.4359 2.5392	0.5846 0.9273 0.9869
Average	5.5170	0.5194	4.7779	0.6031	6.8749	2.2931	3.2750	0.8160	2.3769	0.8329
Ratio BPDE+ AsIII: BPDE	10.6		7.92		2.99		4.01		2.85	

The effects of arsenic on BPDE-damaged DNA have been investigated by many researchers (Tran et al., 2002; Maier et al., 2002; Ho et al., 2002, for example). My experiments, like those of Maier et al., (2002), showed that cell treatment with BPDE (B(a)P in Maier's experiments) and arsenite resulted in increased DNA damage in comparison to cells treated with BPDE only. My experiments used A549 cells, while those of Maier et al., (2002) used Hepa-1 mouse cells, involved pretreatment of cells with arsenite followed by simultaneous treatment of cells with BPDE/B(a)P respectively and arsenite. I followed this simultaneous exposure step further with cellular exposure to cell media containing arsenite only to allow an extended time for repair to occur. In addition, Maier et al., (2002), used B(a)P which introduces another uncertainty. B(a)P is activated by the aryl hydrocarbon hydroxylase and this enzyme has not been consistently implicated in the development of smoking related cancers. The bioactivation of B(a)P to BPDE is less certain than if BPDE is used directly. Regardless of these methodological differences, results from both experiments showed increased adduct levels in cells treated with arsenite and BPDE/B(a)P as compared with cells treated with BPDE/B(a)P only. Adduct levels were at a maximum, 10.6 times greater at 0 hours and 7.92 times greater after 2 hours of incubation in cell media containing arsenic only (see Table 1) and Maier's experiment's, which did not provide this period for repair, demonstrated an 18-fold increased adduct level with 0.1 μM B(a)P and 10 μM arsenite over cell treatment with 0.1 μM B(a)P only (Maier et al., 2002). Interestingly, Maier found that adduct formation was dose-dependent on B(a)P concentration and additionally increased by arsenic concentration (Maier et al., 2002). This result was not tested in my experiments.

Though DNA treated with arsenite and BPDE demonstrate greater complex peak areas than DNA from A549 cells treated with BPDE only, results in both cases indicate that adducts decrease with time (Figures 15 and 16). Likewise, Maier et al., (2002) found that while the presence of arsenite does result in increased adducts, arsenite does not prohibit removal of B(a)P-DNA adducts. As with my results, Maier et al. (2002) found that adduct levels in cells receiving both arsenite and B(a)P did not decrease to the level of adducts found in those cells treated with BPDE only during the time periods tested in these experiments.

Because much is not known about the effects of BPDE and arsenite on A549 cells, some of the parameters used in the experiments involving treatment of cells with BPDE and arsenite were chosen because previous work has produced results that reduce this uncertainty. In Figures 15 and 16 it may be possible to argue that the perceived decrease in adducts is in fact the result of toxic effects from BPDE and arsenite at the 16 and 24 hour data points. The concentration of arsenite used in this experiment (100 μg/L) has been shown to result in low A549 cytotoxicity in experiments by Mei et al., 2002 (submitted for publication). Mei et al., (2002) found that after 24 hours of exposure to 100 μg/L of sodium arsenite approximately 90% of cells survived. So while there is an approximately 10% reduction in viable cells after 24 hours of exposure, this reduction can not totally account for the approximate 2.5-fold reduction in complex peak area seen at 24 hours when compared to the area at 4 hours. As mentioned earlier cytotoxicity can also result from high BPDE concentration. Cytotoxicity is the likely cause for increased adduct concentration seen in A549 cells treated to 20 μM BPDE (not shown). This is

demonstrated by the apparent lack of adduct removal at 24 hours and the appearance of the cells when viewed under the microscope prior to DNA extraction results.

In order to ensure that arsenic was present in the cell when BPDE was added, cells that would be exposed to BPDE and arsenite simultaneously were first pretreated with arsenite for 24 hours. Hartwig et al., (1997) showed pretreatment to be a necessary step to ensure that arsenite will be taken up by the cells. Using human fibroblasts, Hartwig et al. exposed VH16, VH25, and XPC fibroblasts to a 10 μM concentration of arsenite for periods of up to 24 hours and found that after 16 hours the intracellular arsenite concentration did not increase (Hartwig et al., 1997). A 24 hour pretreatment period was chosen also based on results from Mei et al, (submitted for publication) mentioned above, which demonstrated a 90% cell survival rate for A549 cells exposed to 100μg/L arsenite.

While many components of my experimental method were similar to those of other studies. Many of these do not diminish uncertainties that may affect my results. The responses of different cell lines to a specific carcinogen may vary greatly. For example, Mei et al., (2002) found that repair-proficient cells, A549 and GM43, increased cellular levels of p53 in response to 24 hour exposure to arsenite (50-1000 μg/L) while AT2BE and AT5B1 fibroblast cells showed no increase in p53 levels. In the same paper, Mei et al., (2002) found greatly different cell survival percentages among some human fibroblasts. FA1196 and AG06040 fibroblasts showed minimums of approximately 80% and 50% survival respectively after 24 hours exposure to 300 μg/L of arsenite. A549 cells

showed about 70% survival under these conditions. In contrast to results showing an increase in adducts with exposure to BPDE/B(a)P and arsenite, Ho et al., (2002) using the human lung adenocarcinoma cell line CL3 treated with benzo(a)pyrene (0 to 2 μM) and arsenite (5, 10 μM) found that arsenite pretreatment lessened B(a)P cytotoxicity. The above examples demonstrate that responses to an exposure such as BPDE and arsenite can vary according to the cell line used.

Some possible explanations for the differences between cells exposed to BPDE only and those exposed to both BPDE and arsenite follow. Results by Hartwig et al., (1997) have suggested that arsenite interferes with the incision step in NER that allows the bulky adduct to be removed by decreasing the rate of excision. Arsenic's effect then would be to decrease the rate of repair and not to cease excision of the DNA adduct. In application to results seen in Figure 16, one can see that the increase in adducts observed in the cells treated with BPDE and arsenic may be related to a slower rate of repair; as is evident in Table 3 and in Figures 16, repair is occurring throughout the 24 hour period investigated. Likewise, repair of BPDE damage in cell treated with BPDE only, resulting from a relatively low level of exposure, does not appear to be impaired and damage appears to be lower and/or repair is progressing at an adequate rate (Figure 15).

Another consideration is the role of DMSO in the level of DNA damage as evidenced by the complex peak area. DMSO, a free radical scavenger, was used to dissolve BPDE used in this work. Work by Liu et al., (2001) has shown that DMSO reduces damaging ROS caused by arsenite. In my work, DMSO was used in cell media

containing both arsenite + BPDE as well as media containing BPDE only; both groups received the same volume of DMSO. The effect of DMSO on BPDE stability and on the cell was not investigated and results for BPDE treated cells show less damage than BPDE + arsenite treated cells. So, the effects of oxidative damage from arsenite and the effects of scavenging of resulting free radicals by DMSO can not be determined from these experiments. Perhaps an experiment involving cells treated with BPDE dissolved in DMSO and another suitable solvent and arsenite along with control cells treated with BPDE dissolved in DMSO and another solvent may provide a better answer to this question.

In light of the varied responses of cell lines to DNA damaging agents, it is obvious that a generalization to the response of an organism to chemical insult can not be made easily. As mentioned in the introduction, factors such as genetic predisposition, race, and exposure frequency and duration can provide some guidelines but are not necessarily determinants of disease occurrence. Animal studies may possibly allow further insight into the effect of BPDE and arsenite on DNA. Tran et al., (2002) injected Sprague-Dewley rats with 10 mg/kg arsenite with or without 800 μg of benzo(a)pyrene. They found that rats in both groups slaughtered on day 5 (out of 1, 3, 5, 10, 27 days) showed maximum adduct formation. By day 10, B(a)P (in the B(a)P-only exposure group) adducts had decreased approximately initial levels while those rats exposed to both arsenite and B(a)P showed approximately the same number of adducts as seen on day 5. Interestingly, while the B(a)P + arsenite addicts persisted they did not reach the higher adduct levels demonstrated in rats receiving B(a)P only. For obvious reasons, it is

not possible to perform these experiments with humans; while *in vivo* results provide results that are applicable to the whole organism, these results do not necessarily indicate a similar human response.

An investigation into the metabolism of BPDE and arsenite in the A549 cell specifically may remove some uncertainty and provide a better understanding of the cellular response of this particular cell line. In addition to this, further investigation into cytotoxicity, the exposure concentration-repair relationship, as well as profile of the stages of the A549 cell cycle represented in a cell plate in response to and for the duration of exposure to a carcinogen may allow further insight into the cellular response to damage. In addition, cellular uptake and metabolism of BPDE and arsenic would provide clues about the results obtained. Finally, an investigation into oxidative mechanisms induced by arsenic and the potential of DMSO to decrease such damage would be useful.

Chapter 4. Conclusion and future work

In light of the many differences between individuals' personal health and environmental exposure levels and also the great range of experimental results, much work still needs to be done to understand the effects of arsenic on human health. The health of many millions of people depends on an improved understanding of arsenic health effects. The metabolism of arsenic, an important key to understanding arsenic, is gradually being elucidated. With these new revelations come new questions. The contributions of variations such as ethnic factors, exogenous factors (e.g. dietary and

living habits), exposure differences, and genetic factors are still very important to an individual's response to chronic arsenic exposure. Further work should include an investigation into the effects of different arsenic species on the A549 cell line. Experiments which investigate the effects of BPDE in combination with arsenite and MMA^{III} on A549 cells would be interesting in view of new results on arsenic metabolism. It would also be very interesting to know more about the A549 cellular response to arsenic and BPDE. Effect on cell cycle, replication, and metabolic products including reactive oxygen species may decrease the uncertainty in experiments with this cell line. Additionally, epidemiological studies which investigate correlations of health effects and MMA^{III} intake in urine samples for example, may contribute to the understanding of this toxic metabolite and its effect on health. In addition to this, the development of accessible technologies and speed of education to reduce risks among those exposed would greatly improve life for many exposed individuals.

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